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HPLC/DAD Intercomparison on Phytoplankton Pigments (HIP-1, HIP-2, HIP-3 and HIP-4)

Canuti, E., Ras, J., Grung, M., Röttgers, R., Costa Goela, P., Artuso, F., Cataldi, D.

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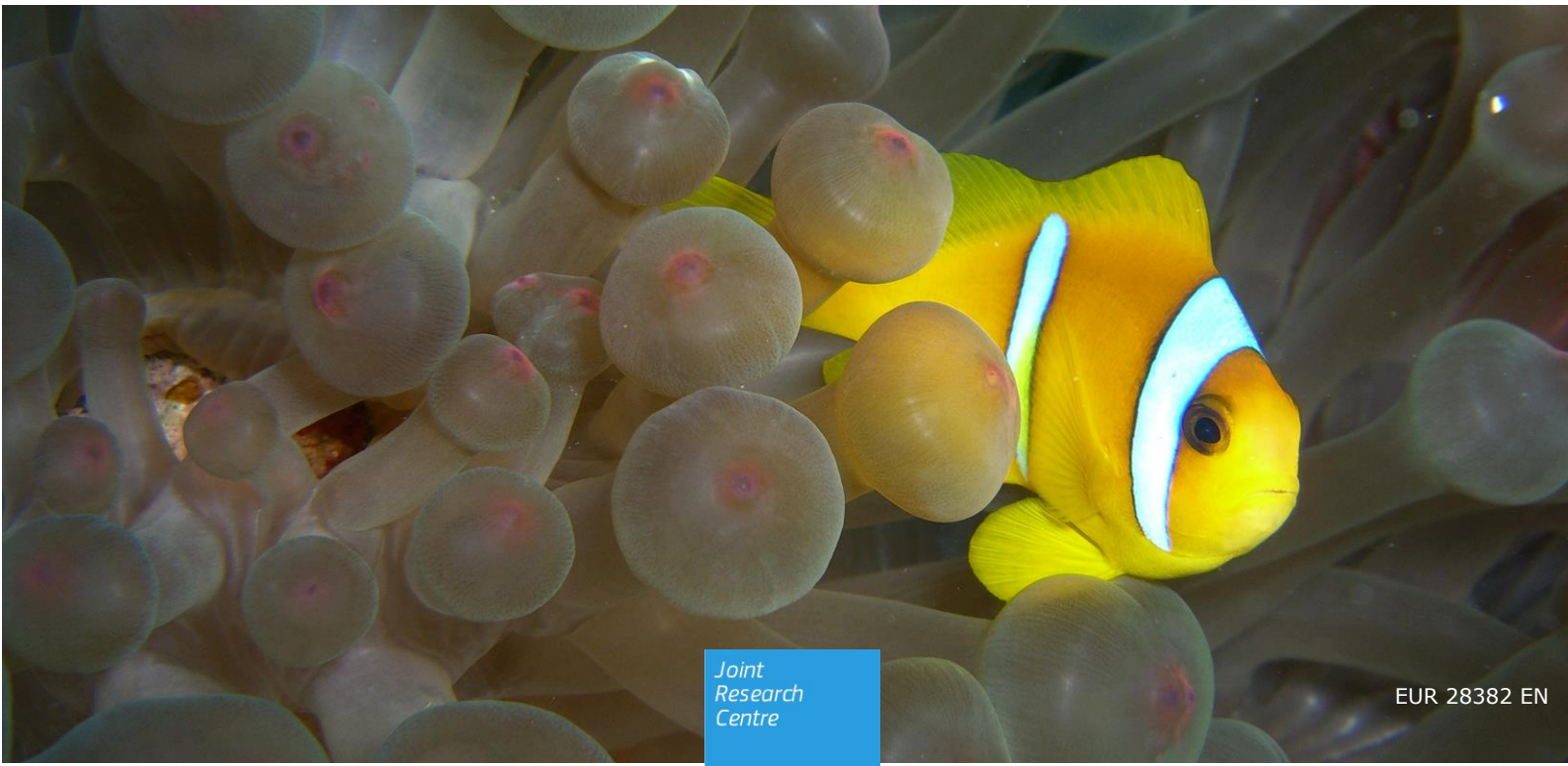

Joint Research Centre

EUR 28382 EN

Contact information
Name: Elisabetta Canuti
Address: DG Joint Research Centre, 21027 Ispra (VA), Italy
Email: elisaebtta.canuti@jrc.ec.europa.eu
Tel.: +39 0332 78 5221

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Authors

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Abstract

From 2009 to 2015, in the context of the MERIS (Medium Resolution Imaging Spectrometer) validation activities, the JRC Marine Optical Laboratory organised four HPLC Intercomparison exercises for Phytoplankton Pigment measurements (HIP-1, HIP-2, HIP-3 and HIP-4), involving seven European accredited and reference laboratories.

The objectives of these intercomparison exercises were: creating a reference community at European level for phytoplankton pigment analysis capable of supporting satellite data validation; quantifying single laboratory uncertainties; improving and maintaining the quality of results for a single laboratory with time and quantifying the differences among European laboratories applying published methods.

The four intercomparisons have confirmed that chlorophyll *a* uncertainties requirement for satellite data validation activities (25% in oligotrophic water) are achievable for laboratories applying in HPLC phytoplankton pigment analysis.

1. Introduction

Satellite data product validation and bio-optical algorithm development require availability of high quality *in-situ* measurements of chlorophyll *a*. Intercomparison exercises performed for HPLC methods demonstrated that an uncertainty lower than 6 % for the determination of chlorophyll *a* and within 25 % for the other ancillary pigments is achievable (Hooker et al. 2005).

The present report is an assessment of the results from four HPLC Intercomparisons of Phytoplankton Pigments (HIP-1, HIP-2, HIP-3 and HIP-4) organized within the framework of MERIS validation activities. From 2009 to 2015, these intercomparisons have involved seven European laboratories: the Danish DHI Institute for Water and Environment (DHI), the French CNRS *Laboratoire d'Océanographie de Villefranche* (LOV), the Joint Research Centre (JRC) Marine Optical Laboratory of the European Commission Laboratory, the Norwegian Institute for Water Research (NIVA), the Portuguese Centre for Marine and Environmental Research - University of Algarve (CIMA), the German *Helmholtz-Zentrum Geesthacht* (HZG) and the Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA).

The objectives of these exercises were: *i.* the quantification of uncertainties across European laboratories utilizing assessed methods, for a set of reference pigment standards and natural samples representing different environmental and trophic conditions; and *ii.* the creation of a European reference community for HPLC phytoplankton pigment analysis.

2. The JRC Marine Optical Laboratory

The JRC Marine Optical Laboratory provides reliable measurements within given uncertainties of pigments concentration in natural marine waters. Regular participation in Round Robin and intercalibration exercises ensures the quality of the measurements performed.

From 2001 to 2007, the JRC took part in three SeaWiFS HPLC Analysis Round-Robin Experiments (SeaHARRE) organized by NASA (National Aeronautics and Space Administration): SeaHARRE-1, SeaHARRE-3 and SeaHARRE-4 (Hooker et al. 2000, 2009 and 2010). The method adopted by the JRC for SeaHARRE-1 and for SeaHARRE-3 was derived from the original Wright method (JGOFS, 1994). In June 2007, the JRC adopted the Van Heukelem and Thomas (2001) method as modified for SeaHARRE-3 (Van Heukelem and Thomas, 2009). This method allows the separation and the quantification of a larger amount of taxonomic relevant pigments than the previously used (Wright et al. 1991) and, in particular, the indipendent quantification of monovinyl and divinyl chlorophyll *a*. The Van Heukelem and Thomas method has been successfully applied to a wide range of pigment concentrations from the oligotrophic Central Pacific Ocean (SeaHARRE-3, Hooker et al. 2009) to the eutrophic coastal South African waters (SeaHARRE-2, Hooker et al. 2005). Other advantages of the van Heukelem method are the use of an internal standard, Vitamin E acetate (tocopherol acetate), that is identified at a separate wavelength with respect to other compounds and additionally through the mobile phase creates less buffer crystal deposits in the HPLC circuits with respect to the Wright method.

The JRC took part in SeaHARRE-4 with the new method while it was still being set up. The performance obtained was better than during the SeaHARRE-3 round robin. During the following HIP intercomparisons, the JRC applied the fully implemented Van Heukelem method, with a well-established routine and quality certified ISO-9001.

Note that during the HIP-3 exercise, a part of the procedures (sample extraction and injection) was performed by a different operator who was new to this kind of analysis and technique.

Even in this case, the JRC Marine Optical Laboratory performed measurements at a quantitative level.

3. HIP-1, HIP-2, HIP-3 and HIP-4 exercises

In 2008, within the framework of MERIS validation activities the JRC Marine Optical Laboratory, planned an HPLC Intercomparison exercise on Phytoplankton Pigments (HIP-1) in order to continue the validation process of the method implemented. The objective of the first HIP exercise was to quantify uncertainties across European reference or certified laboratories that use the same assessed method implemented at the JRC. The participants in the exercise were asked to produce their own performance metrics evaluation as defined in Hooker et al. (2005) and to perform analysis at 'routine' level. The exercise was drafted over a set of mixed pigment standards and a series of 12 natural samples in triplicate. The natural samples were collected during different field campaigns and covered a broad range of concentrations (e.g. chlorophyll *a* concentrations varied from 0.4 to 2.4 mg/m^3), a typical range for the JRC analysis.

The HIP-1 exercise was launched at the beginning of 2009, and involved three laboratories: the Danish DHI Institute for Water and Environment (DHI), the French CNRS *Laboratoire d'Océanographie de Villefranche* (LOV) and the European Commission Joint Research Centre Marine Optical Laboratory (JRC). The LOV reported problems on the HPLC system (see Annex I) during the exercise. They received an extra batch of natural samples, however these results are not discussed in the present report.

HIP-2, the second HPLC intercomparison performed under the JRC responsibility, started in 2010 and was considered as a sequel to the HIP-1. The invitation to participate in this exercise was extended to other European laboratories involved in MERIS validation activities and using different analytical methods for phytoplankton pigment determination. HIP-2 therefore involved five European laboratories: the three former participants to HIP-1 and, the Norwegian Institute for Water Research (NIVA) and the Portuguese Centre for Marine and Environmental Research - University of Algarve (CIMA). The latter two laboratories applied methods derived from Jeffrey et al. (1997).

The samples distributed for HIP-2 comprised 12 batches of replicate natural samples (25 mm, GF/F) collected in 2010 during two field campaigns in the Adriatic Sea (July 2010) and in the Ligurian Sea (August 2010), with chlorophyll *a* concentrations varying from 0.08 to 4 mg/m^3. A set of mixed pigment standards were also included. The CIMA institute decided to join the exercise at a later stage and only received 11 batches. Three laboratories (DHI, LOV and JRC) received 6 extra batches of triplicates of 47 mm GF/F filters, matching 6 of the 25 mm batches. The objective of this additional exercise was to compare results derived from the extraction of different sample filter sizes.

In 2011, the JRC continued the intercomparison exercises by organising HIP-3 involving six laboratories: JRC, DHI, LOV, NIVA, CIMA and the German *Helmholtz-Zentrum Geesthacht* (HZG). Three analytical methods were compared in HIP-3. All participants were asked to perform the extraction exercise on different sizes of sample filters (25 and 47 mm): the same exercise had already been proposed during HIP-2, but only to a restricted group of three laboratories. In total, 12 batches of replicate natural samples (25 mm) collected in 2011 during three field campaigns (chlorophyll *a* concentrations ranged from 0.15 to 5.5 mg/m^3), 6 batches of 47 mm natural sample triplicates (corresponding to 6 batches of 25 mm filter replicates) and six mixed pigment standards were distributed. CIMA never submitted its results due to a problem with the HPLC system that occurred between December 2011 and February 2012. The JRC did not analyse the W series for the HIP-3 exercise because of a problem during the extraction phase of the samples. NIVA did not receive the F series, due

to a mistake during the sample packaging and delivering. HZG did not report 19'-butanoyloxyfucoxanthin and peridinin pigments in the natural samples but only reported them for the mixed standards.

Due to a problem that emerged with the mixed standard analysis during HIP-3, a fourth exercise was planned in 2013 and launched in 2014. HIP-4 started in 2013, involving 5 laboratories which applied 2 different analytical methods. HIP-4 was mainly focused on standards and sample storage, preservation and handling. The 5 participants to HIP-4 were: JRC, DHI, LOV, NIVA and the Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA). The samples, 4 series of triplicates with a mean chlorophyll *a* concentration of 1.5 mg/m^3, were collected during a field campaign in the Adriatic Sea (December 2013), and were distributed in September 2014 with 9 standard mixes representing two different concentrations levels.

During HIP-4, NIVA performed the analysis with the newly implemented Van Heukelem & Thomas method. The HPLC system used by NIVA during the HIP-4 intercomparison was equipped with a standard injection loop. The low volume of sample injected, affected the limit of detection. Because of this NIVA was not able to quantify the low concentration mixed standard and quantified only three Primary Pigments for the natural samples. ENEA encountered a problem with the -80 C freezer where the samples and the mixes were preserved. The freezer defrosted and both samples and mixed standards were found at room temperature: the number of days at room temperature is unknown. ENEA reported a series of problems in implementing the method on a new HPLC system (column and pre-column blocking). These problems were not solved during HIP-4. The ENEA performance metrics submitted for HIP-4, refer to the system status before these problems were observed.

During the 4 HIP exercises, 744 natural samples were distributed among 7 laboratories of 7 different European countries and 4 different methods were compared giving a comprehensive overview of the HPLC phytoplankton pigments analysis activities in support of satellite data validation in Europe.

Table 1. List of participants in HIP-1, HIP-2, HIP-3 and HIP-4.

Laboratories Acronyms	***Laboratories***	***HIP-1***	***HIP-2***	***HIP-2 (secondary exercise)***	***HIP-3***	***HIP-4***
D	**DHI**, Danish Institute for Water and Environment, Denmark	Y	Y	Y	Y	Y
L	**LOV**, CNRS *Laboratoire d'Océanographie de Villefranche*, France	Y	Y	Y	Y	Y
J	**JRC**, Marine Optical Laboratory, Joint Research Center, European Commission	Y	Y	Y	Y (not for the extraction exercise)	Y
C	**CIMA**, Centre for Marine and Environmental Research - University of Algarve		Y		Y (results not submitted)	
N	**NIVA**, Norwegian Institute for Water Research, Norway		Y		Y	Y
H	**HZG**, Helmholtz-Zentrum Geesthacht, Germany				Y	
E	**ENEA,** Italian National Agency for New Technologies, Energy and Sustainable Economic Development, Italy					Y

Table 2. Summary of the activities for the HIP exercises

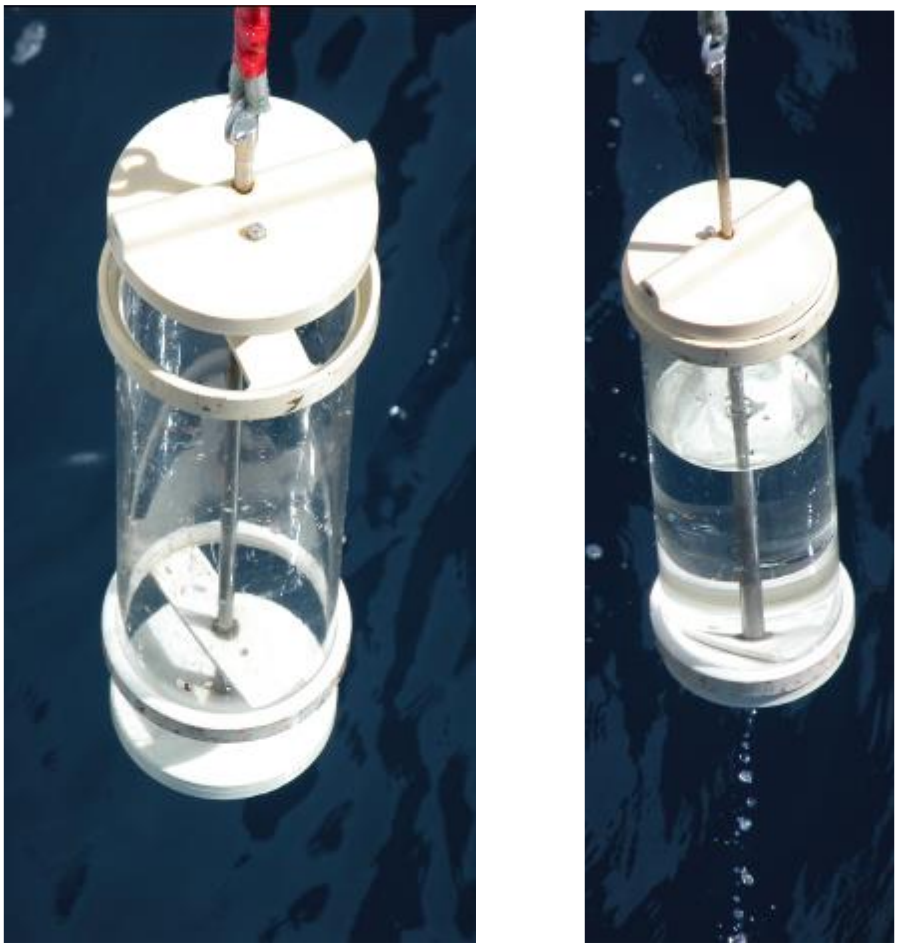
Exercise	Laboratories	Country	Responsible	Method compared	Natural samples distributed
HIP-1	*D*	Denmark	L. Schlüter, lsc@dhigroup.com and M. Allerup, mea@dhigroup.com	Van Heukelem and Thomas (2001)	108
	L	France	J. Ras, jras@obs-vlfr.fr		
	J	European Commission	E. Canuti, elisabetta.canuti@jrc.ec.europa.eu		
HIP-2	*D*	Denmark	L. Schlüter, lsc@dhigroup.com and M. Allerup, mea@dhigroup.com	Van Heukelem and Thomas (2001)	270
	L	France	J. Ras, jras@obs-vlfr.fr		
	J	European Commission	E. Canuti, elisabetta.canuti@jrc.ec.europa.eu		
	C	Portugal	P. Costa Goela, priscila.goela@gmail.com	Jeffrey et al. (1997)	
	N	Norway	M. Grung, merete.grung@niva.no		
HIP-3	*D*	Denmark	L. Schlüter lsc@dhigroup.com and M. Allerup, mea@dhigroup.com	Van Heukelem and Thomas (2001)	306
	L	France	J. Ras, jras@obs-vlfr.fr		
	J	European Commission	E. Canuti, elisabetta.canuti@jrc.ec.europa.eu		
	C	Portugal	P. Costa Goela, priscila.goela@gmail.com	Jeffrey et al. (1997)	
	N	Norway	M. Grung, merete.grung@niva.no		
	H	Germany	R. Röttgers, rroettgers@hzg.de	Zapata et al. (2000)	
HIP-4	*D*	Denmark	L. Schlüter, lsc@dhigroup.com and M. Allerup, mea@dhigroup.com	Van Heukelem and Thomas (2001)	60
	L	France	J. Ras, jras@obs-vlfr.fr		
	J	European Commission	E. Canuti, elisabetta.canuti@jrc.ec.europa.eu		
	N	Norway	M. Grung, merete.grung@niva.no and A. Kringstad, alfhild.kringstad@niva.no		
	E	Italy	F. Artuso, florinda.artuso@enea.it and D. Cataldi	Vidussi et al. (1996)	

4. Natural samples: sample collection, preservation and distribution

The field samples for HIP-1, HIP-2, HIP-3 and HIP-4 were prepared following the same sampling protocol.

The water was collected using a 3.5 litres plastic sampling bottle (Fig. 1) during the execution of a CTD (SeaBird, US) cast and, for HIP-1 and HIP-3, of an AC9 (Wet Labs, US).

Fig. 1. JRC sampling bottle. The sampler is made of Plexiglas and PVC plastic. The sampler is deployed 1 m below the surface for collecting water.



The water was stored at room temperature in the dark in 10 L polyethylene bottles (Kartell, IT) until filtration. The total amount of collected water was stirred (Heidolph, UK) in a stainless steel container (50 L) and evenly distributed among the samples. The filtration volumes were defined according to the value of the attenuation coefficient at 412 nm measured during water sampling. The filtration volumes were measured with 2 L plastic cylinders (Duran, DE). The filtrations were carried out on a stainless steel manifold (Sartorius, DE) under mild vacuum (Millipore vacuum pump, US) on 25 mm or 47 mm GF/F, 0.7 µm (Whatman, DE) filters. The collected samples were wrapped in aluminium foil, labelled and immediately stored in liquid nitrogen. After their arrival at the JRC laboratories, the samples were transferred to temperature controlled freezer at - 80°C (Thermo 900 -86C ULTC, Thermo, US).

The field sample replicates for HIP-1 were collected during 3 different field campaigns from January to April 2009: the AAOT_D4, AAOT_D5 campaigns at the Acqua Alta Oceanographic Tower (AAOT) situated in the Adriatic Sea (lat. 45°19', long. 12°30') and the LCSV09 cruise performed in the Ligurian Sea (Fig.2).

Fig. 2. Spatial distribution of replicates collected during the LCSV09 cruise (12-23 March 2009) on-board the NURC R/V Alliance.

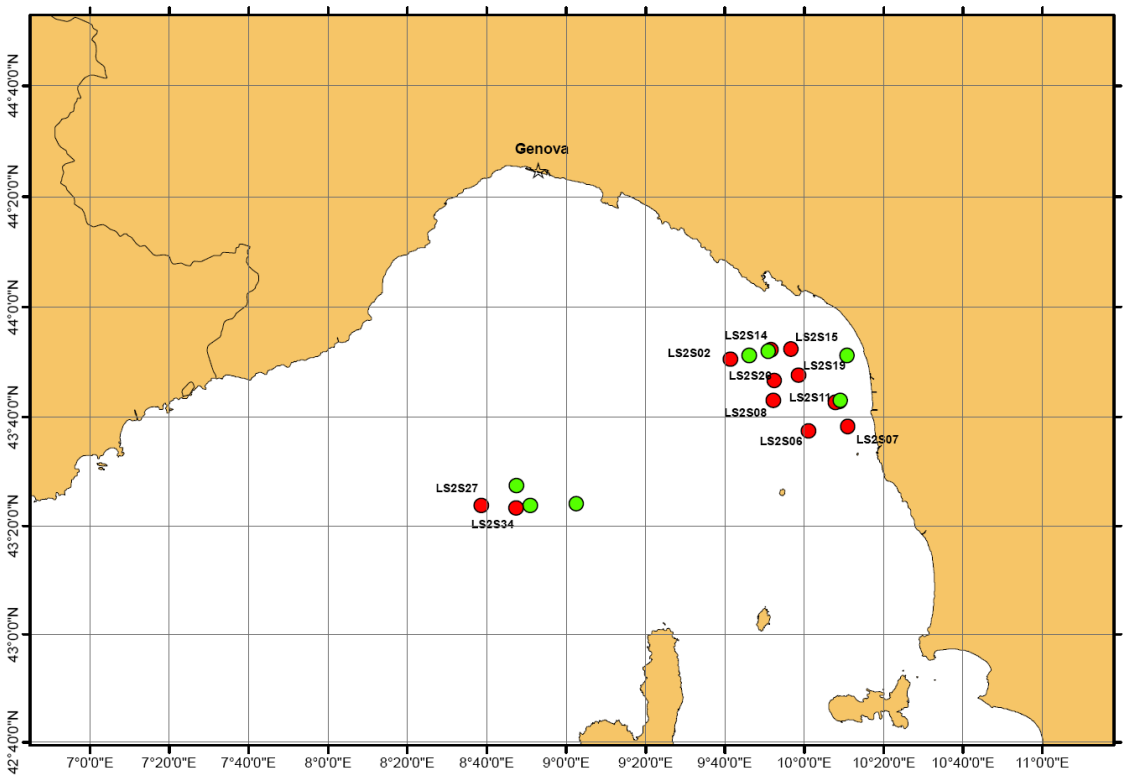


Table 3. List of HIP-1 samples distributed. The filter size was 25 mm for all the batches with the exception of C and Y that were 47 mm.

Sample Code	*LAT.*	*LONG.*	*Campaign Code*	*Storage in Liquid Nitrogen*	*Storage at – 80 C*	*Attenuation coeff. 412 [nm/m] (determined by AC9)*
G	43.84130	9.68940	LS2(S02)	13.03.09	22.03.09	0.45
H	43.63745	10.18243	LS2(S07)	14.03.09	22.03.09	1.5
I	43.62306	10.01775	LS2(S06)	14.03.09	22.03.09	0.75
L	43.71036	10.13119	LS2(S11)	15.03.09	22.03.09	1.8
M	43.71630	9.86960	LS2(S08)	15.03.09	22.03.09	0.3
N	43.87300	9.94420	LS2(S15)	16.03.09	22.03.09	0.9
P	43.87100	9.86030	LS2(S14)	16.03.09	22.03.09	0.65
Q	43.77600	9.87470	LS2(S26)	17.03.09	22.03.09	2
V	43.39489	8.64390	LS2(S27)	18.03.09	22.03.09	1
Z	43.38800	8.79020	LS2(S34)	19.03.09	22.03.09	0.8
C (47 mm)	45 19'	12 30'	D5(S100)	28.01.09	30.01.09	---
Y (47 mm)	43.71630	10.15201	LS2(S44)	21.03.09	22.03.09	0.1

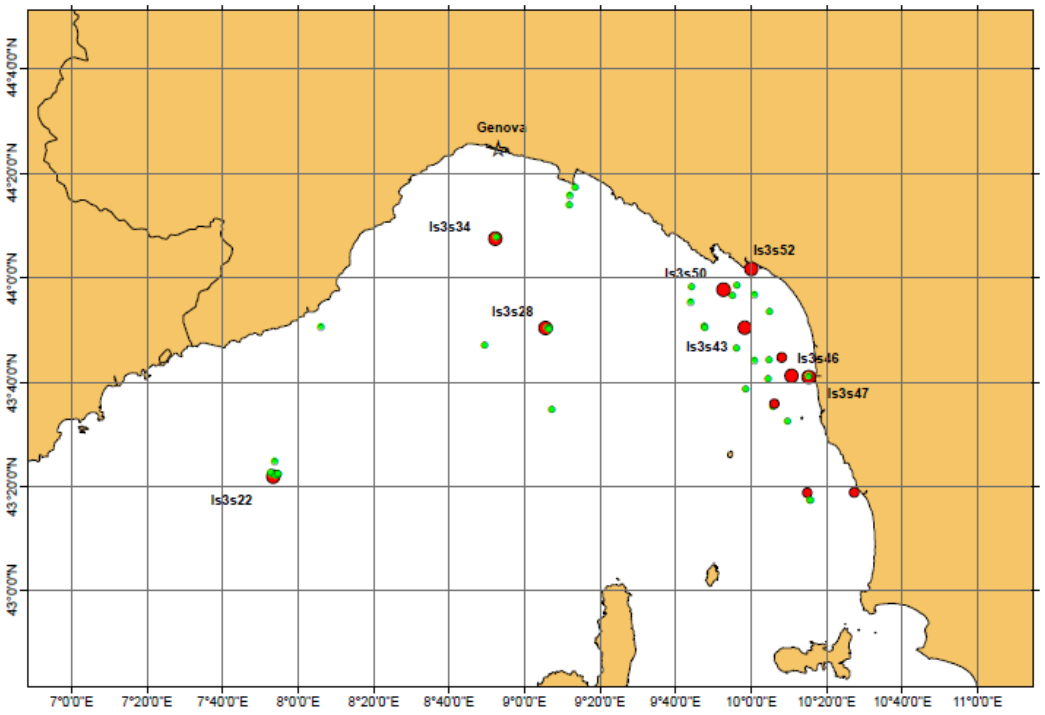
The samples collected are representative of different bio-optical conditions: coastal well as oligotrophic and eutrophic waters were sampled. In total, 23 series of replicates were collected and amongst these 12 were chosen to be distributed among HIP participants.

On the 10th September 2009, the 12 samples series of triplicates were packed in dry ice and delivered to *D* and *L* laboratories, together with the standard mixes (Table 3).

HIP-2 natural samples were collected during the ARC 10 (Adriatic Sea, AAOT, July 2010) and LCSV10 (Ligurian Sea, R/V Alliance, August 2010) oceanographic cruises for various sampling conditions. 25 series of 25 mm filters in triplicate were collected, in addition to 10 series of 47 mm replicates.

On the 29th September 2010, the 12 series of triplicate samples were packed in dry ice and delivered together with the standard mixes (8 vials of DHI mix 107 batch), to all five participants (Table 4). *D* and *L* also received and analysed 6 series of 47 mm triplicates matching 6 series of 25 mm triplicates that were distributed for the main exercise.

Fig. 3. Spatial distribution of replicates collected during the LCSV10 cruise (15 August - 3 September 2010) on board the NURC R/V Alliance.



HIP-3 natural samples were collected during the IB1 (Iberian Sea, R/V Almirante Gago Coutinho, April 2011), BL4-5-6 (Black SeaSea, R/Vs Mare Nigrum and Akademik, July 2011) oceanographic cruises and during a campaign performed at the AAOT (Adriatic Sea, September 2011). 16 series of triplicates of 25 mm filters diameter were collected plus 6 series of 47 mm replicates.

On the 21th September, 2011 the 12 series of triplicate 25 mm samples and 6 series of 47 mm triplicates (matching 6 series of 25 mm triplicates) were packed in dry ice and delivered, together with the standard mixes (6 vials of DHI mix 108 batch), to all six participants (Table 5).

Table 4. HIP-2 natural samples. The filter size is 25 mm. When two sample codes are present, the second one refers to the 47 mm series.

Sample Code	*LAT.*	*LONG.*	*Campaign Code*	*Storage in Liquid Nitrogen*	*Storage at – 80 C*
B/C	45 19′	12 30′	AAOT	21.07.11	24.07.11
D/E	45 19′	12 30′	AAOT	21.07.11	24.07.11
G/H	45 19′	12 30′	AAOT	22.07.11	24.07.11
I/L	45 19′	12 30′	AAOT	22.07.11	24.07.11
T	43.21908	7.534354	LS3(S24)	26.08.10	04.09.10
V	43.5043	9.5551	LS3(S28)	28.08.10	04.09.10
Z	44.75528	8.52284	LS3(S34)	29.08.10	04.09.10
AA	43.50477	9.5835	LS3(S43)	31.08.10	04.09.10
AH	43.412847	10.107551	LS3(S46)	01.09.10	04.09.10
AG/AF	43.41378	10.15275	LS3(S47)	01.09.10	04.09.10
AM	43.578384	9.526651	LS3(S50)	02.09.10	04.09.10
AI/AL	44.017906	10.0013	LS3(S52)	02.09.10	04.09.10

Table 5. HIP-3 natural samples. The filter size is 25 mm. When two sample codes are present, the second one refers to the 47 mm series.

Sample Code	*LAT.*	*LONG.*	*Campaign Code*	*Storage in Liquid Nitrogen*	*Storage at – 80 C*	*Attenuation coeff. 412 [nm/m] (determined by AC9)*
A	39.49702	-9.63628	IB1(s15)	02.04.11	19.04.11	0.20
B	38.46153	-9.54558	IB1(s22)	06.04.11	19.04.11	0.35
C	39.31203	-9.56355	IB1(s59)	14.04.11	19.04.11	0.30
D	44.84386	30.28331	BL4(s13)	02.07.11	27.07.11	2.4
F/E	44.49605	29.49846	BL4(s25)	04.07.11	27.07.11	2.8
H/G	42.32468	28.92945	BL5(s07)	09.07.11	27.07.11	0.5
L/I	43.52028	28.62757	BL5(s21)	11.07.11	27.07.11	0.6
N/M	42.49415	28.6647	BL6(s06)	13.07.11	27.07.11	0.5
Q/P	42.69274	31.66452	BL6(s18)	15.07.11	27.07.11	0.4
R	42.94461	33.33422	BL6(s24)	16.07.11	27.07.11	0.4
S	43.56223	30.33188	BL6(s42)	19.07.11	27.07.11	0.4
W	45 19′	12 30′	AAOT	14.09.11	16.07.11	0.20

HIP-4 samples were all collected during the AAOT_G3 measurement campaign (Adriatic Sea, 12 December 2013) and filtered at the JRC laboratories in Ispra (Table 6). The samples were from the same water batch preserved in two different ways and filtered in different quantities. The filters used were 25 mm in diameter for all the batches.

The standard mixes (mix-1 112 and mix-2 102) were purchased from DHI and stored at -20°C after their arrival. The freezer temperature was regularly checked (bi-weekly) until expedition.

On the 9th September 2014, the 4 series of triplicates were packed in dry ice together with 6 vials of mix-1 112 and mix-2 102 pigment standards and delivered to the 5 HIP-4 participants. The shipping boxes (Sonoco Thermosafe, USA) were identical for all laboratories: same inner chamber volume and equal amount of dry ice were used for each pack. All the packages were delivered within 48 h.

Table 6. HIP-4 natural samples. The filter size was 25 mm.

Sample Code	*Campaign Code*	*Filtering date*	*Storage*	*Filtered amount (mL)*
A	AAOT_G3	13.12.13	Filtered at arrival at the JRC laboratory	600
B	AAOT_G3	13.12.13	Filtered at arrival at the JRC laboratory	300
C	AAOT_G3	16.12.13	Water stored at room temperature for 3 days	500
D	AAOT_G3	16.12.13	Water stored at 4°C for 3 days	500

5. Laboratory Methods

The participants in HIP-1, HIP-2, HIP-3 and HIP-4 adopted methods derived from the following published methods: Van Heukelem and Thomas (2001), Jeffrey et al. (1997), Zapata et al. (2000) and Vidussi et al. (1996). The methods are described in detail in Hooker et al. (2005) for *D*, Ras et al. (2008) for *L*, Hooker et al. (2010) for *J*, Jeffrey et al. (1997) for *C* and *N* (HIP-2 and -3), Van Heukelem and Thomas (2001) for *N* (HIP-4), Zapata et al. (2000) for *H* and Vidussi et al. (1996) for *E*.

The extraction procedures are summarized in Table 7. Both acetone and methanol were used as extraction solvents. The parameters that differed the most were the extraction time, from 2 to 24 hours, and the extraction temperature that varied, from room temperature till -20°C. Filter disruption was performed by sonication in all cases, except for *H.* Sample clarification was mainly done by filtration and/or centrifugation.

All laboratories used an HPLC with a DAD detector acquiring signals at different wavelengths according to their methods (Table 8). The stationary phases of the columns were essentially composed of C8 and C18. The mobile phases (Table 8 and 9) did vary a lot in terms of solvents and solvent gradients, even amongst laboratories applying the same initial method. The two-phase and three-phase solvent gradients were evenly adopted by all laboratories (Table 9).

The main characteristics of the extraction procedures and method implementation are summarized in Table 7, 8 and 9.

All the laboratories calculated the individual pigment concentrations (C_{pi}) by applying the same calculation:

1) $$C_{Pi} = \frac{V_x \cdot \hat{A}_c \cdot \hat{A}_{Pi} \cdot R_{Pi}}{V_f \cdot V_c \cdot \hat{A}_s}$$

where $\hat{A}_{Pi}$ and R_{Pi} are the peak area and the response factor of pigment P_i, respectively; V_x is the extraction volume; V_f is the volume of the water filtered for each sample; V_c is the amount of sample injected onto the column; $\hat{A}_c$ is the peak area of the internal standard in the extraction solvent; and $\hat{A}_s$ is the peak area of the internal standard in the sample.

C and *N* (for HIP-2 and 3) applied the Latasa et al. (1996) dichromatic equations to quantify the monovinyl chlorophyll *a* and divinyl chlorophyll *a* contributions.

The effective Limit of Detection (LOD_{eff}), when reported, is calculated as:

2) $$LOD_{eff} = LOD \cdot \frac{V_x}{V_f \cdot V_c}$$

For statistical analysis, this value is used instead of the term "*not quantified"* or replaces the Limit of Quantitation (LOQ). LOQ represents 10 times the signal to noise ratio, while the LOD represents 3 times the signal to noise ratio. Few laboratories (*L* for HIP-2 exercise, *H* for HIP-3 and *N* for HIP-4) did not report any LOD or LOQ. When a pigment was not quantified by any of the laboratories, it was considered as not present.

Table 7. Extraction procedures adopted for HIPs by the involved laboratories.

	Laboratories	***Extraction Solvent***	***Extraction Volume***	***Soaking Time***	***Disruption***	***Disruption Time***	***Clarification***	***Extraction Time and Temperature***
Φ 25 mm GF/F (0.7 µm pore size)	*D*	Acetone 95 % containing 0.0025 µg/mL of Vitamin E acetate	3 mL	20-24 hours	Sonication bath	10 min in ice cold	0.2 µm Teflon syringe filter	20-24 hours
	L	Methanol 100% with Vitamin E acetate	3 mL	1 hour + 1 hour after sonication	Sonication probe	10 sec	GF/F filter under mild vacuum	2 hours (-20 C)
	J	Acetone 100% containing 0.0025 µg/mL of Vitamin E	2.5 mL + 150 µl of double distilled water	1 hour + 3.5 to 4 hours after sonication	Sonication	90 second in cold ice	0.2 µm Teflon syringe filter	4.5 – 5 hours (-20 C)
	C	Acetone 90%	3 mL	_ _	Sonication bath	12 min in ice	Centrifuge (15 min at 6000 rpm)	15 – 18 hours (4 C)
	N	Acetone 90%	3 mL	10 min + 4 hours after sonication	Sonication	30 sec in ice	Centrifuge	4 hours (room T)
	N (HIP-3)	Acetone 90%	2 mL	10 min + 4 hours after sonication	Sonication	30 sec in ice	Centrifuge	4 hours (-20 C)
	N (HIP-4)	Acetone 90%	5 mL	10 min + 20 hours after sonication	Sonication bath	1 min in ice	Teflon syringe filter	20 hours (-20 C)
	H	Acetone 100%	5 mL	24 hours	_ _	_ _	0.2 µm Spartan 13A filter	24 hours (-30 C)
	E	Acetone 100%	2 mL	24 hours	Sonication bath	10 min in ice cold	Centrifuge (20 min at 4000 rpm, 4 C) and 0.2 µm Nylon syringe filter	24 hours (4 C)
Φ47 mm GF/F (0.7 µm pore size)	*D*	Acetone 95 % containing 0.0025 µg/mL of Vitamin E acetate	5 mL	20-24 hours	Sonication bath	10 min in ice cold	0.2 µm Teflon syringe filter	20-24 hours
	L	Methanol 100% with Vitamin E acetate	8 mL	1 hour + 1h after sonication	Sonication probe	10 sec	GF/F filter under mild vacuum	2 hours
	L (HIP-3)	Methanol 100% with Vitamin E acetate	6 mL	1 hour + 0.25 hour between sonication+ 1 hour after sonication	Sonication probe	10 sec + 10 sec in cold ice	GF/F filter under mild vacuum	2.25 hours (-20 C)
	J	Acetone 100% containing 0.0025 µg/mL of Vitamin E	5 mL	1 hour + 3.5/4 hours after sonication	Sonication	120 second in cold ice	0.2 µm Teflon syringe filter	4.5 – 5 hours
	N	Acetone 90%	3 mL + 540 µl of water	10 min + 4 hours after sonication	Sonication	30 sec in ice	Centrifuge	4 hours (-20 C)
	H	Acetone 100%	5 mL	24 hours	_ _	_ _	0.2 µm Spartan 13A filter	24 hours (-30 C)

Table 8. HPLC methods implemented during HIPs: main parameters.

Laboratories	*Method adopted*	*Instrument*	*Injection Volume (µL)*	*Acquired Wavelengths (nm)*		*Stationary Phase*	*Mobile Phase*
D	Van Heukelem et al. 2001	Shimadzu , SPD-M10A VP-DAD	143	450	Rest of the quantified pigments	ZORBAX XDB-C8 3.5 µm particle size, 4.6x150mm at 60 C.	**A:** 70:30 Methanol: 0.4M TBAA **B:** Methanol
				665	Chlorophyll *a* and derived		
L	Van Heukelem et al. 2001 (modified)	Agilent, HPLC 1100 (HIP-1); Agilent, HPLC 1200 (HIP-2)	125	450	Carotenoids, Chlorophylls *c* and *b*	ZORBAX XDB-C8 3.5 µm particle size, 3x150mm at 60 C.	**A:** 70:30 Methanol: 0.4M TBAA **B:** Methanol
				676	Chlorophyll *a* and derived		
				770	Bacteriochlorophyll *a*		
J	Van Heukelem et al. 2001	Agilent, HPLC 1100	135	450	Rest of the quantified pigments	ZORBAX XDB-C8 3.5 µm particle size, 4.6x150mm at 60 C.	**A:** 70:30 Methanol: 0.4M TBAA **B:** Methanol **C:** Acetone
				665	Chlorophyll *a* and derived		
C	Jeffrey et al. 1997 (modified)	Agilent, LC 1200	50	436	Phaeopigments, Chlorophyllide *a* and Chlorophyll *a*	Alltech Altima C18 3.5 µm particle size, 4.6x150mm	**A:** 80:20 Methanol: 0.5M Ammonium Acetate **B:** 90:10 Acetonitrile: Water **C:** Ethyl Acetate
				450	Carotenoids, Chlorophyll*b*, Chlorophyll*c2* and *c3*		
N *(HIP-2 and -3)*	Jeffrey et al. 1997	Waters 2695 HPLC Waters 2996 Diode array	100	410	Pheophorbide, Pheophytin a	Thermo hypersil C18 ODS 5 µm particle size 4.0×250mm	**A:** 80:20 Methanol: 0.5M Ammonium Acetate **B:** 90:10 Acetonitrile: Water **C:** Ethyl Acetate
				436	Phaeopigments, Chlorophyllide *a* and Chlorophyll *a*		
				450	Carotenoids, Chlorophyll *b*, Chlorophyll *c2* and *c3*		
N (HIP-4)	Van Heukelem et al. 2001	Waters 2695, HPLC Waters 2996 Diode array	25	410	Pheophorbide, Pheophytin a	Waters symmetry C8 3.5 µm particle size 4.6×150mm	**A:** 70:30 Methanol: 0.028M TBAA (pH 6.5) **B:** Methanol
				436	Phaeopigments, Chlorophyllide *a* and Chlorophyll *a*		
				450	Carotenoids, Chlorophyll *b*, Chlorophyll *c2* and *c3*		
H	Zapata et al. 2000	HPLC Jasco		434	Rest of the quantified pigments	Guard column: Waters Symmetry C8 3.5 µm particle size 2.1x10 mm Column: Waters Symmetry C8 3.5 µm particle size 4.6x150 mm	**A:** 50:25:25 Methanol: Acetonitrile: 0.25M pyridine solution **B:** 80:20 Acetonitrile: Acetone
				668	Pheophytin *a*, Pheophorbide *a*		
E	Vidussi et al. 1996	Agilent, HPLC 1260	100	440	All quantified pigments	Supelco, Ascentis series C8, 3µm particle size, 3x100mm	**A:** 70:30 Methanol: 0.5M Ammonium Acetate **B:** Methanol

Table 9. Mobile phase gradients: the solvents for each laboratory are detailed in Table 8.

C				
Time (min)	**Flow rate (ml/min)**	**%A**	**%B**	**%C**
0	1	100	0	0
4	1	0	100	0
18	1	0	20	80
21	1	0	100	0
24	1	100	0	0
29	1	100	0	0

D			
Time (min)	**Flow rate (ml/min)**	**%A**	**%B**
0	1.1	95	5
27	1.1	5	95
34	1.1	5	95
35	1.1	0	100
38	1.1	0	100
39.5	1.1	95	5
46	1.1	95	5

E			
Time (min)	**Flow rate (ml/min)**	**%A**	**%B**
0	1	75	25
1	1	50	50
15	1	0	100
18.5	1	0	100
19	1	75	25

H			
Time (min)	**Flow rate (ml/min)**	**%A**	**%B**
0	1	100	0
18	1	60	40
22	1	0	100
34	1	0	100
37	1	100	0

J				
Time (min)	**Flow rate (ml/min)**	**%A**	**%B**	**%C**
0	1.1	95	5	0
22	1.1	5	95	0
24.5	1.1	5	95	0
24.75	1.3	5	65	30
25.75	1.3	5	65	30
25.85	1.3	5	65	30
26.1	1.1	95	5	0
29.1	1.1	95	5	0

L			
Time (min)	**Flow rate (ml/min)**	**%A**	**%B**
0	0.55	10	90
22	0.55	95	5
27	0.55	95	5
28	0.55	95	5
33	0.55	100	0

N (HIP-2 and 3)				
Time (min)	**Flow rate (ml/min)**	**%A**	**%B**	**%C**
0	1	100	0	0
4	1	0	100	0
18	1	0	20	80
21	1	0	100	0
24	1	100	0	0
29	1	100	0	0

N (HIP-4)			
Time (min)	**Flow rate (ml/min)**	**%A**	**%B**
0	1.1	95	5
2.0	1.1	95	5
13.0	1.1	50	50
17.0	1.1	50	50
25.0	1.1	5	95
27.0	1.1	5	95
29.0	1.1	0	100
31.0	1.1	0	100
33.0	1.1	95	5
37.0	1.1	95	5

6. Pigments

The phytoplankton pigments considered for the intercomparison exercises were the chlorophylls and carotenoids most commonly used in chemotaxonomic and photophysiological studies.

The adopted nomenclature for total chlorophylls and the other pigments was that established by the SCOR (Scientific Committee on Oceanographic Research) Working Group 78 (Jeffrey *et al*. 1997) (Table 10).

Table 10. Pigment definitions and acronyms.

	Primary Pigments (PPig)
TChl *a*	Total Chlorophyll *a* (Chlorophyllide-*a* + Monovinyl Chlorophyll *a* + Divinyl Chlorophyll *a*)
TChl *b*	Total Chlorophyll *b* (Monovinyl Chlorophyll *b* + Divinyl Chlorophyll *b*)
TChl *c*	Total Chlorophyll *c* (Chlorophyll *c1*+ Chlorophyll *c2* + Chlorophyll *c3*)
Car	Carotenes bb-Carotene +be-Carotene
Allox	Alloxanthin
ButFuco	19'-Butanoyloxyfucoxanthin
Diad	Diadinoxanthin
Diat	Diatoxanthin
Fuco	Fucoxanthin
HexFuco	19'-Hexanoyloxyfucoxanthin
Per	Peridinin
Zeax	Zeaxanthin
	Secondary and Tertiary Pigments
MVChl *a*	Monovinyl Chlorophyll *a*
DVChl *a*	Divinyl Chlorophyll *a*
Chlide *a*	Chlorophyllide *a*
Pheo *a*	Pheophorbide *a*
Phy *a*	Pheophytin *a*
Pras	Prasinoxanthin
Viol	Violaxanthin
Neo	Neoxanthin
Lut	Lutein

According to the classification adopted in the SeaHARRE Round Robin exercises, total chlorophylls and carotenes are defined as primary pigments, while pheophorbide *a* and pheophytin *a* are considered as 'secondary pigments', since they are individual pigments that form a primary pigment composed by separate contributions (e.g. total chlorophylls). Secondary and tertiary pigments also included pigments not routinely analysed or separated by the laboratories that performed similar types of analysis (monovinyl chlorophyll *a*, divinyl chlorophyll *a*, chlorophyllide *a*, neoxanthin, violaxanthin, pheophythin *a*, pheophorbide *a*, prasinoxanthin, lutein).

7. Method validation

The methods were evaluated and compared at three different stages with the aim of identifying specific sources of uncertainties.

These uncertainties were calculated according to the guidelines defined in EURACHEM/CITAC Guide (1998).

The first stage was a laboratory self-evaluation. All the participants were required to provide their own performance metrics calculation as defined in Hooker et al. (2005). These criteria, adopted in several Round Robin exercises for HPLC algal pigments analysis (Hooker et al. 2005, 2009, 2010 and 2012), evaluate the method precision and uncertainty over 10 different parameters. The final rank is weighed on parameters that take into account both the laboratory practice and method performance. The threshold for the admission to the HIP exercises was fixed in 'routine performance'. An uncertainty within 15% for the TChl *a* and within 25% for the PPig in natural samples ('semi-quantitative' threshold) was required.

The evaluation of the uncertainties derived from the analytical method implementation was made through the analysis of standard pigment mixes created as artificial mixtures from cultural stocks. The distributed standard mixes were all from the same batch for each exercise: mix 106 batch for HIP-1, mix 107 batch for HIP-2, mix 108 batch for HIP-3 and mixes mix-1 112 and mix-2 102 (low concentration range) for HIP-4. Each mix batch was considered homogenous. The comparison of results from the mixes focused on the HPLC methods themselves, thus excluding the effects of the sample extraction phase and the intrinsic biases of the natural samples.

The third stage was the evaluation of uncertainties derived from the sample collection, handling and extraction procedure. These were calculated through the analysis of natural samples batches (12 batches for HIP-1, HIP-2 and HIP-3, 4 batches for HIP-4): the natural samples were distributed in batches of triplicates.

The statistics applied for the three-step method validation is described in detail in Chapter 8.

8. Results

8.1 Laboratory Validation: performance metrics evaluation

A series of parameters have been defined during the SeaHARRE Round Robin exercises with the intent of giving an overall evaluation of laboratory precision and uncertainty levels. The performances metrics, described in detail in Hooker et al. (2005), are used to define the threshold ('routine' level) for HIP participants. Most of the parameters taken into account could be calculated by the laboratories themselves, with the exception of the precision and uncertainty of the TChl *a* and PPig that are calculated on the distributed 25 mm natural sample replicates (10 batches for HIP-1, 12 batches for the HIP-2 and HIP-3, 4 batches for HIP-4) and for which a 'semi-quantitative' uncertainty level is required. The performance metric

Table 11. Definition of performance parameters.

Acronym	Description	Definition	Calculation Remarks						
			D	*L*	*J*	*C*	*N*	*E*	*H*
$\overline{\xi}_{TChla}$	TChl *a* precision	TChl *a* percent variation for the intercomparison triplicates							
$\left\|\overline{\psi}\right\|_{TChla}$	TChl *a* uncertainty	TChl *a* absolute percent difference calculated on the intercomparison triplicates							
$\overline{\xi}$	PPig precision	Average of PPig percent variation for the intercomparison triplicates	Pigments considered: TChl *a*, TChl *b*, TChl *c*, Per, But Fuco, Hex Fuco, Fuco, Diad, Diat, Allox, Zeax, Car.						
$\left\|\overline{\psi}\right\|$	PPig uncertainty	Average of PPig absolute percent difference calculated on the intercomparison triplicates	the intercomparison triplicates						
$\widehat{R}_S$	Separation: peaks Resolution	Peak resolution calculated for worst of critical pairs	Zeax-Lut and Hex Fuco-Viol-Pras (determined from DHI mix injections)	Zeax-Lut (determined from DHI mix injections)	Zeax-Lut and Hex Fuco-Viol-Pras (determined from DHI mix standard injections)	Zeax-Lut			
$\overline{\xi}_{tR}$	Separation: retention time (t) Precision	average of percent variation of retention times for		TChl *a* and Fuco in 10 natural samples from one sequence	All the quantified compounds on the 3 injections of mix standards in the same sequence	All the quantified compounds on the 3 injections of mix standards in the same sequence	All the quantified compounds on the 3 injections of mix standards in the same sequence		
$\overline{\xi}_{inj}$. Peridin	Injection Precision for Per	percent variation of peak areas for Peri in DHI mix standard	on 3 successive injections of mixed pigments	for Per in DHI mix	on the 3 injections of mix standards in the same sequence				
$\overline{\xi}_{inj}$. Chl a	Injection Precision for TChl *a*	percent variation of peak areas for TChl *a* in DHI mix standard	on 3 successive injections of mixed pigments	of peak areas for TChl *a* in DHI mix	on the 3 injections of mix standards in the same sequence				
$\left\|\overline{\psi}\right\|_{res}$	$\overline{\xi}_{cal}$	Average of the absolute values of deviation from the calibration curve.	For TChl *a*	For TChl *a*	Average for all the PPig	For TChl *a*			
$\overline{\xi}_{cal}$	Calibration Precision		average of the percent variation for the Pipette (acetone) precision	average of the percent variation for the 2 Hamilton syringes used for calibration and for the Eppendorf pipette used to dispense extraction volumes	average of the percent variation for the 2 Hamilton syringes used for calibration and for the Dispense pipette used to dispense extraction volumes	average of the percent variation for dilution devices (3 micro-syringes Hamilton for HPLC) on acetone			

definitions and the acronyms are described in Table 11, including the participants' remarks regarding their computation.

During HIP-1 and 2, via their self-evaluation, all the laboratories demonstrated the capability to perform 'quantitative analysis' according to SeaHARRE-2 score ranking. During HIP-3, *L* improved its performance metrics ('state of art') while *N* was able to perform 'quantitative' and *H* qualified as 'routine' (values calculated only on the basis of submitted results). During HIP-4, *D*, *L* and *J* confirmed the performance during HIP-3 and *N* with the newly implemented Van Heukelem method obtained 'quantitative' level, as during HIP-3. *E* performed 'semi-quantitative', but provided evidence of a well operating HPLC instrument as it was before the analysis of the HIP-4 mixes and natural samples (during HIP-4, *E* reported several operational problems with the HPLC system).

Results from the different laboratories are summarized in Table 12a, b, c and d.

Most of the participants are accredited or reference laboratories and this is confirmed by the overall good performance during HIP intercomparisons or other round robin exercises.

Before the HIP exercises, *J* was classified as 'routine' during the SeaHARRE-3 round robin using a modified Jeffrey method, and 'semi-quantitative' during SeaHARRE-4 when the new Van Heukelem method was implemented, with different instrument operators. During the HIP intercomparisons, the performance of *J* was 'quantitative' with the Van Heukelem & Thomas method being fully implemented and a well assessed laboratory routine in place. The method change, from JGOFS (1994) applied during SeaHARRE-3, to Van Heukelem & Thomas, adopted since 2008, resulted in an improved laboratory performance. The Van Heukelem & Thomas method solved analytical problems encountered with the previous JGOFS method, such as the separation of the DVChl *a* - MVChl *a* critical pair, the crystallization and deposition on the HPLC circuit of Ammonium Acetate (present in the JGOFS mobile phase) and the identification of internal standards (trans-β-apo-8´-carotenal) at the same wavelength as other pigments. During HIP-3, *J* was qualified as 'quantitative' even though the natural sample extractions were performed by an operator who was new to this technique and had little experience.

*D*uring HIP-1, 2, 3 and 4, *D* confirmed the 'quantitative' level demonstrated with the same implemented method during the SeaHARRE-3, SeaHARRE-4 and SeaHARRE-5 experiments.

L improved from the 'quantitative' level obtained in SeaHARRE-3, SeaHARRE-4, HIP-1 and HIP-2, to the 'state of art' level during HIP-3 and HIP-4 and in SeaHARRE-5.

N evaluated its performance for the first time during HIP-2 obtaining a 'quantitative' level. *N* confirm to 'quantitative' during HIP-3 and it scored at 'quantitative' level again during HIP-4 with the newly implemented Van Heukelem & Thomas method.

Table 12a. Performance Metrics calculation table: the SeaHARRE-2 (SH2) scored rank is given in green. The notation n.s. indicates "not submitted" data. The performance metrics parameters calculated on natural samples for each exercise are highlighted in yellow.

Table 12a. HIP-1 Performance metrics table.

	TChl *a*		**PPig**		**Separation**		**Injection** $\overline{\xi}_{inj}$		**Calibration**		PERFORMANCE
	$\overline{\xi}$	$\lvert\overline{\psi}\rvert$	$\overline{\xi}$	$\lvert\overline{\psi}\rvert$	$\widehat{R}_S$	$\overline{\xi}_{tR}$	Per	Chl *a*	$\lvert\overline{\psi}\rvert_{res}$	$\overline{\xi}_{cal}$	
D	5.9	4.4	6.1	4.5	1.2	0.05	0.22	0.41	0.4	0.57 (Ac)	**3.3**
SH2-rank	**2**	**4**	**2**	**4**	**3**	**3**	**4**	**4**	**4**	**3**	**quantitative**
L	6.0	5.7	5.7	4.2	1.32	0.03	1.51	0.26	2.3	1.3 (MeOH)	**2.9**
SH2-rank	**2**	**3**	**2**	**4**	**2**	**4**	**4**	**4**	**2**	**2**	**quantitative**
J	6.4	4.9	8.6	6.4	1	0.03	1.27	0.15	3.4	1.33 (Ac)	**2.8**
SH2-rank	**2**	**4**	**1**	**4**	**2**	**4**	**4**	**4**	**1**	**2**	**quantitative**

Table 12b. HIP-2 Performance metrics table.

	TChl *a*		PPig		Separation		Injection $\overline{\xi}_{inj}$		Calibration		PERFORMANCE
	$\overline{\xi}$	$\lvert\overline{\psi}\rvert$	$\overline{\xi}$	$\lvert\overline{\psi}\rvert$	$\hat{R}_S$	$\overline{\xi}_{tR}$	Per	Chl *a*	$\lvert\overline{\psi}\rvert_{res}$	$\overline{\xi}_{cal}$	
D	3.0	2.2	6.4	5.5	1.2	0.05	0.13	0.66	0.4	0.57 (Ac)	**3.4 quantitative**
SH2-rank	**3**	**4**	**2**	**4**	**3**	**3**	**4**	**4**	**4**	**3**	
L	3.7	5.0	5.4	7.3	1.04	0.05	1.8	0.85	0.1	0.86	**3.1 quantitative**
SH2-rank	**2**	**4**	**2**	**4**	**2**	**3**	**4**	**4**	**4**	**2**	
J	6.3	4.0	12.0	9.0	1	0.03	1.27	0.15	2.7	1.3 (Ac)	**2.7 quantitative**
SH2-rank	**1**	**4**	**1**	**4**	**2**	**4**	**4**	**4**	**1**	**2**	
C	6.0	4.6	2.1	2.7	2.15	0.6	0.87	0.91	0.98	0.28	**3.3 quantitative**
SH2-rank	**1**	**4**	**4**	**4**	**4**	**0**	**4**	**4**	**4**	**4**	
N	6.7	5.0	8.6	6.4	0.88	0.05	4.65	0.72	1.07	0.07	**2.6 quantitative**
SH2-rank	**1**	**4**	**1**	**4**	**1**	**3**	**2**	**4**	**3**	**3**	

Table 12c. HIP-3 Performance metrics table.

	TChl *a*		PPig		Separation		Injection $\overline{\xi}_{inj}$		Calibration		PERFORMANCE
	$\overline{\xi}$	$\lvert\overline{\psi}\rvert$	$\overline{\xi}$	$\lvert\overline{\psi}\rvert$	$\hat{R}_S$	$\overline{\xi}_{tR}$	Per	Chl *a*	$\lvert\overline{\psi}\rvert_{res}$	$\overline{\xi}_{cal}$	
D	5.2	3.8	8.4	6.2	1.2	0.05	0.33	0.5	0.89	0.57	**3.4 quantitative**
SH2-rank	**2**	**4**	**2**	**4**	**3**	**3**	**4**	**4**	**4**	**4**	
L	2.9	3.9	4.6	3.4	1.03	0.04	0.8	0.2	0.9	0.68	**3.5 state of art**
SH2-rank	**3**	**4**	**3**	**4**	**2**	**4**	**4**	**4**	**4**	**3**	
J	7.0	5.1	10	8.2	1	0.03	1.27	0.15	2.7	1.3 (Ac)	**2.7 quantitative**
SH2-rank	**1**	**4**	**1**	**4**	**2**	**4**	**4**	**4**	**1**	**2**	
N	3.3	2.4	18	13.4	0.72	0.05	6.25	0.16	2.64	0.33	**2.6 quantitative**
SH2-rank	**2**	**4**	**0**	**3**	**1**	**3**	**1**	**4**	**4**	**4**	
H	4.8	3.4	17.9	13.8	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**0.9 routine**
SH2-rank	**2**	**4**	**0**	**3**	**0**	**0**	**0**	**0**	**0**	**0**	

Table 12d. HIP-4 Performance metrics table. (*) Information recovered from the previous exercises (no new are submitted). (**) Values on PPig were calculated on the basis of the three PPig submitted for the natural samples.

	TChl *a*		PPig		Separation		Injection $\overline{\xi}_{inj}$		Calibration		PERFORMANCE
	$\overline{\xi}$	$\lvert\overline{\psi}\rvert$	$\overline{\xi}$	$\lvert\overline{\psi}\rvert$	$\hat{R}_S$	$\overline{\xi}_{tR}$	Per	Chl *a*	$\lvert\overline{\psi}\rvert_{res}$	$\overline{\xi}_{cal}$	
D	3.9	2.9	6.4	4.7	1.2*	0.05*	0.33*	0.5*	0.89*	0.57*	**3.4 quantitative**
SH2-rank	**2**	**4**	**2**	**4**	**3**	**3**	**4**	**4**	**4**	**4**	
L	2.5	1.9	5.9	4.2	1.03*	0.10	0.8*	0.33	0.4	0.3	**3.5 state of art**
SH2-rank	**4**	**4**	**3**	**4**	**2**	**2**	**4**	**4**	**4**	**4**	
J	2.7	2.1	7.4	5.5	1	0.07	2.0	1.3	2.7	1.3 (Ac)	**3.0 quantitative**
SH2-rank	**4**	**4**	**2**	**4**	**2**	**3**	**4**	**3**	**2**	**2**	
N	5.6	4.1	4.2**	3.2**	1.37	0.29	3.8	2.7	2.5	0.26	**2.8 quantitative**
SH2-rank	**1**	**4**	**3**	**4**	**3**	**0**	**3**	**3**	**3**	**4**	
E	48.5	33.8	12.7	9.2	2.3	0.025	2.5	3.5	2.92	1.4	**2.2 semi quantitative**
SH2-rank	**0**	**0**	**1**	**4**	**4**	**4**	**3**	**2**	**2**	**2**	

C also evaluated its performance metrics for the first time during HIP-2, obtaining 'quantitative' performance level.

E evaluated its performance metrics during HIP-4 with the Vidussi method, obtaining 'semi-quantitative' measurements.

H never submitted the required performance metrics parameters and its 'routine' performance was calculated on the basis of the submitted results on HIP-3 natural samples alone.

8.2 HPLC Analytical Method Validation: analysis of mixed standards

The statistical approach used for comparing the results is that adopted on several HPLC phytoplankton pigments method intercomparisons and Round Robin exercises (Claustre et al. 2004; Hooker et al. 2000, 2005, 2009, 2010 and 2012).

For each single pigment concentration ($\widetilde{C}_{Pi}^{L_j}$) of the mixed standard analysed by each laboratory (L_j) the average concentration ($\overline{C}_{Pi}^{L_j}$) over the number of mixed standard analysis performed by each laboratory (N^{L_j}) was calculated:

3) $$\overline{C}_{Pi}^{L_j} = \frac{1}{N^{L_j}} \sum_1^{N^{L_j}} \widetilde{C}_{Pi}^{L_j}$$

The standard deviation ($\sigma_{Pi}^{L_j}$) and the percent variation coefficient ($\xi_{Pi}^{L_j}$) are defined as:

4) $$\sigma_{Pi}^{L_j} = \sqrt{\frac{1}{N^{L_j}} \sum_1^{N^{L_j}} (\widetilde{C}_{Pi}^{L_j} - \overline{C}_{Pi}^{L_j})}$$

5) $$\xi_{Pi}^{L_j} = 100 \frac{\sigma_{Pi}^{L_j}}{\overline{C}_{Pi}^{L_j}}$$

The average ($\overline{C}_{Pi}^{A}$) of each pigment was calculated across all the mixed standards analysed (N) by the laboratories part of the reference subset (A).

6) $$\overline{C}_{Pi}^{A} = \frac{1}{N} \sum_1^{N} \widetilde{C}_{Pi}^{A}$$

The absolute unbiased percent difference, $|\psi|_{Pi}^{L_j}$, was calculated for each pigment of a single laboratory with respect to the average value across the reference laboratory subset.

7) $$|\psi|_{Pi}^{L_j} = 100 \left| \frac{\widetilde{C}_{Pi}^{L_j} - \overline{C}_{Pi}^{A}}{\overline{C}_{Pi}^{A}} \right|$$

The difference between one laboratory and the average value should not exceed 25% for more than 3 PPig (15 % for TChl *a*) for the laboratories to be considered as part of the same reference subset. *N* was excluded from the reference subset for the HIP-2 exercise due to differences higher than 25 % for ButFuco, Fuco, Per and TChl *c. E* was excluded from the subset in HIP-4 for exceeding 25% for 5 PPig: TChl *a,* Car, HexFuco, Per, Zeax.

8.2.1 HIP-1 results for mixed standards

The HIP-1 exercise required, at least, 3 analyses of the distributed mixed standards.

D delivered 9 analysis of the standard, *L* 14 and *J* 4.

The TChl *b* values of *D* were not averaged with those of *L* and *J* because considered not homogeneous. *D* only reported MVChl *b* for the mixed standard while *J* and *L* reported TChl *b* values without splitting them into MVChl *b* and DVChl *b*.

Only for the mixed standard analysis, *D* reported a value of Caro lower by an order of magnitude with respect to *L* and *J*. This did not occur for the natural samples. As for TChl *b*, *D* values for Caro were not averaged with those of *L* and *J*.

Table 13. Mixed standard (mix 106) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated as a function of the method for the PPig and MVChl *a*. The overall average for each pigment is given in the ***A*** row.

$\|\psi\|$ mix 106	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	4.4	- -	4.4	- -	1.1	12.3	0.3	3.3	4.5	0.4	11.7	3.1	1.5	4.3
L	5.1	26.4	11.1	7.9	1.4	0.9	6.2	9.2	6.0	2.3	3.3	11.6	3.0	7.2
J	0.7	15.4	15.5	1.9	2.5	13.3	5.9	5.9	10.6	1.9	8.4	14.6	1.5	8.1
A	3.4	20.9	10.3	4.9	0.7	8.8	4.1	6.1	7.1	1.5	7.8	9.8	2.0	6.7

Table 14. Mixed standard (mix 106) percent error ($\xi_{Pi}^{L_j}$) determined for different laboratories for and MVChl *a*. The overall average of each pigment is given in the ***A*** row.

ξ mix 106	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	0.8	1.2	0.8	2.1	1.1	1.7	0.9	1.2	1.1	1.5	1.3	0.9	1.0	1.2
L	6.0	8.2	2.5	7.2	5.3	6.2	4.4	5.0	3.7	4.6	6.0	4.6	6.0	5.3
J	0.3	0.9	7.1	0.6	0.6	1.2	1.2	0.7	16.2	1.7	4.5	0.1	0.2	2.9
A	2.5	3.4	3.5	3.3	2.3	3.0	2.2	2.3	7.0	2.6	3.9	1.9	2.4	3.1

The laboratories exhibited good agreement for the mixed standard measurements. The uncertainty of laboratories was 3.4 % for TChl *a*, and 6.7 % for PPig (Table 13).

The average precision was 2.5 % for TChl *a* and 3.1 % for PPig (Table 14) this confirmed that good performance metrics are a prerequisite for obtaining close results on the mixed standard quantification.

Table 15. Mixed standard (mix 106) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated for different laboratories for the secondary and tertiary pigments. The overall average for each pigment is given in the ***A*** row.

$\|\psi\|$ mix 106	Chlide *a*	DVChl *a*	MVChl *a*	Phy *a*	Neo	Prasi	Viola	Lut
D	28.6	11.3	2.4	12.8	14.3	6.8	17.4	6.4
L	18.4	8.2	2.2	8.4	9.5	6.3	10.6	8.1
J	- -	3.2	2.3	0.4	0.9	6.6	2.1	14.1
A	15.7	7.6	2.3	7.2	8.2	6.6	10.1	9.5

Results of the secondary and tertiary pigments (Chlide *a*, MVChl *a*, DVChl *a*, Pheo *a*, Neo, Pras, Viol, Lut) partially confirm those obtained for the PPig. The MVChl *a* uncertainty was 2.3% while the precision was 2.4% (Table 15 and 16). However, for the other pigments the average uncertainty and precision seemed poorer than those obtained for PPig.

Table 16. Mixed standard (mix 106) variation coefficient ($\xi_{Pi}^{L_j}$) determined for each laboratory for secondary and tertiary pigments. The overall average for each pigment is given in the ***A*** row.

ξ mix 106	Chlide *a*	DVChl *a*	MVchl *a*	Phy *a*	Neo	Prasi	Viola	Lut
D	2.0	1.1	1.3	1.7	1.1	0.9	1.0	1.2
L	14.9	7.1	6.0	5.5	4.9	5.8	6.0	9.2
J	- -	3.6	2.6	1.2	1.4	0.2	0.2	0.6
A	8.5	3.9	3.3	2.8	2.5	2.3	2.4	3.7

8.2.2 HIP-2 results for mixed standards

D and *L* provided 8 analysis of the mixed standard. *J, C* and *N* provide 9.

The mixed standards were analysed for three different injection sequences to determine the method stability with time and with different mobile phases, as well as to analyse the mix at least three times.

For the mixed standard, *D* only reported MVChl *b*, and not TChl *b,* like all the other laboratories. Their values are therefore not presented in Table 17.

The reference subset for HIP-2 comprises three laboratories implementing methods derived from the Van Heukelem & Thomas method and one laboratory using the Jeffrey method: all the methods compared during HIP-2 are represented in the subset. *N* was excluded due to strong differences (higher than 25%) for four PPig with respect to the average.

The uncertainty, within the reference subset, is 4.5% for the PPig, and 3.5 % for the TChl *a* (Table 17). Within respect to the subset, *N* uncertainties are lower than 15% for TChl *a*. On average, the precision for the reference group is 1.2% for the PPig and 1.1% for TChl *a* (Table 18).

Table 17. Mixed standard (mix 107) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated as a function of the method for the PPig, MVChl *a* and DVChl *a*. In red, values higher than 25%. The overall average for each pigment for the reference subset is given in the ***A*^{s}** entries.

$\|\psi\|$ mix 107	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*	PPig
D	2.6	- -	0.2	1.4	0.6	6.9	3.4	1.8	3.2	2.9	2.3	10.2	14.5	4.1	3.2
L	1.9	11.9	2.5	6.0	0.9	8.6	3.3	3.0	9.1	3.7	24.8	13.4	6.3	4.8	7.4
J	5.2	4.1	0.2	3.8	3.2	3.8	2.7	4.0	3.1	2.3	9.6	15.9	0.7	2.4	4.8
C	4.6	7.8	2.9	8.3	2.9	11.8	2.8	2.7	9.0	4.2	17.5	12.6	7.4	6.6	7.3
N	13.5	27.3	67.8	11.6	41.8	0.6	9.1	1.5	29.3	34.7	57.1	15.1	--	--	25.8
***A*^{s}**	3.5	7.9	1.5	4.9	1.9	7.8	3.0	2.9	6.1	3.3	13.5	13.0	7.2	4.5	4.5

The results for the secondary and tertiary pigments are reported from three laboratories (*D*, *L* and *J*) and the pigments quantified are: Chlide *a*, MVChl *a*, DVChl *a*, Neo, Pras, Viol and Lut (Table 19 and 20), while *C* only reported the MVChl *a*, DVChl *a*. The uncertainty and precision obtained for the secondary and tertiary pigments are close to those obtained for the PPig. The MVChl *a* uncertainty is 4.5%, with a 1.6% variation coefficient.

Table 18. Mixed standard (mix 107) percent error ($\xi_{Pi}^{L_j}$) for the associated method corresponding to the PPig, MVChl a and DVChl a. The overall average concentration for each pigment for the reference subset is given in the As entries.

ξ mix 107	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*	PPig
D	0.8	0.1	3.1	0.8	0.6	0.7	1.8	5.1	5.8	0.6	4.5	3.7	0.7	0.8	2.3
L	1.0	0.5	0.9	1.1	1.0	0.8	0.8	0.8	1.3	1.0	1.2	0.9	1.3	1.0	1.0
J	1.3	0.7	1.5	1.6	0.4	0.4	0.3	0.2	0.7	0.7	1.7	0.4	0.5	1.7	0.8
C	1.3	1.5	1.0	3.1	1.1	0.9	1.0	1.4	0.7	0.6	1.0	1.2	1.6	1.5	1.2
N	0.6	0.9	4.4	1.9	4.8	4.7	2.1	2.4	1.4	4.5	6.0	1.8	--	--	3.0
***A*^{s}**	1.1	0.8	1.6	1.8	0.8	0.6	0.9	0.9	1.4	0.7	2.1	1.6	1.2	1.1	1.3

Table 19. Mixed standard (mix 107) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated as a function of the method for the secondary and tertiary pigments.*C* laboratory is included in the QA subset average, because they reported MVChl a and DVChl a . The overall average for each pigment is given in the ***A*** entries.

$\|\psi\|$ mix 107	Chlide *a*	DVChl *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	3.9	14.5	4.1	0.0	0.1	7.3	1.5
L	10.0	6.3	4.8	0.5	8.2	9.6	2.1
J	6.1	0.7	2.4	0.5	8.1	2.2	3.6
C	- -	1.6	1.5	- -	- -	- -	- -
A	6.7	7.2	4.5	0.4	5.4	6.4	2.4

Table 20. Mixed standard (mix 107) percent errors ($\xi_{Pi}^{L_j}$) associated to each method, corresponding to the secondary and tertiary pigments. The overall average for each pigment is given in the ***A*** entries.

ξ mix 107	Chlide *a*	DVChl *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	0.0	0.7	0.8	0.8	1.1	0.8	0.7
L	2.0	1.3	1.0	0.3	0.7	0.3	0.6
J	0.2	0.5	1.7	0.3	0.3	0.3	0.8
C	- -	1.6	1.5	- -	- -	- -	- -
A	0.7	1.2	1.1	**0.5**	**0.7**	**0.5**	**0.7**

8.2.3 HIP-3 results for mixed standards

D submitted 9 measurements of the standard, *L* 14, *H* 5, *N* 9, and *J* 4.

It was requested that the mixed standards should be analysed in three different injection sequences to consider the method stability in time and in changing of mobile phases.

In comparison with the two previous exercises, significant differences were observed between laboratories, including those which obtained close results during the HIP-1 and 2 exercises.

N measured 3 different mix 108 standard vials and reported the results of 9 injections. There is a remarkable difference between one group of 6 injections and another group of 3 (see Table 21)

N reported that the TChl *a* standard control measurements, performed in parallel with the mix 108 measurements, were consistent throughout the different injection sequences even when it was not the case with the mixed standard.

J tested two out of six mixed standard vials by spectrophotometry (Lambda 35 Perkin Elmer, US) and verified the TChl *a* content by using the trichromatic equation (Jeffrey and Humprey, 1975). The TChl *a* quantified in the two mixed standards (4.2 and 4.9 mg/m^3) was lower than the amount certified from DHI for this mix (6.29 mg/m^3). These two standards were shipped in dry ice to DHI in June 2013 for further verification. *J* measured two other mixed standards by spectrophotometry and sent them to *L* laboratories for further verification. The results (Table 22) suggested non-homogeneity in the mixes distributed for the HIP-3 exercise.

Table 21. Mixed standard (mix 108) PPig, MVChl a and DVChl a average concentration values (mg/m^3) as a function of the different methods. *N*** and *N** are respectively the *N* average onto 2 vials measurements (6 measurements) and onto a single vial measurement (3 measurements). *L*$^+$ are the *L* data resubmitted in 2016.

mg/m^3 mix 108	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*
D	6.03	1.50	0.59	0.40	0.32	0.18	0.17	0.21	0.14	0.29	0.51	0.33	1.13	4.78
L	3.88	1.59	0.41	0.26	0.23	0.13	0.12	0.15	0.11	0.20	0.26	0.22	0.49	3.31
L$^+$	5.61	2.29	0.57	0.37	0.33	0.19	0.17	0.21	0.16	0.29	0.37	0.31	0.70	4.79
J	4.72	1.10	0.55	0.36	0.34	0.19	0.18	0.20	0.15	0.30	0.42	0.41	0.69	3.81
H	5.34	2.14	0.68	0.30	0.37	0.20	0.22	0.20	0.16	0.31	0.37	0.32	0.53	4.08
N (all)	5.95	2.18	0.51	0.37	0.26	0.17	0.24	0.22	0.17	0.30	0.36	0.52	1.04	3.59
*N***	6.62	2.29	0.49	0.42	0.25	0.19	0.23	0.20	0.16	0.34	0.33	0.52	1.05	4.20
*N**	4.63	1.94	0.54	0.27	0.30	0.12	0.25	0.26	0.19	0.23	0.42	0.52	1.03	2.36

Table 22. Mixed standard (mix 108) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated as a function of the method for the PPig, MVChl a and DVChl a. No reference subset was defined. *N*** and *N** are respectively the *N* averages for 2 vial measurements (6 injections) and for a single vial measurement (3 injections). Values higher than 25% (15% for the TChl a) are in red. The overall average for each pigment is given in the ***A*** entries.

$\|\psi\|$ mix 108	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*	PPig
D	16.8	11.9	7.7	18.3	5.3	3.4	8.6	7.1	4.1	3.6	32.8	8.3	45.6	22.1	10.7
L	24.9	6.6	25.2	23.1	24.3	25.3	35.5	23.5	24.7	28.6	32.3	38.9	36.9	15.4	26.1
J	9.0	35.4	0.4	6.5	11.8	9.2	3.2	2.0	2.7	7.1	9.4	13.9	11.1	2.7	9.4
H	3.4	25.7	24.1	11.2	21.7	14.9	18.3	2.0	9.6	10.7	3.6	11.1	31.7	4.2	13
N (all)	15.2	28.1	6.9	9.5	14.5	2.3	29.0	12.2	16.4	7.1	6.3	44.4	34.0	8.3	16
*N***	28.1	34.8	10.8	24.9	19.1	10.9	23.1	3.6	10.3	21.4	13.3	43.1	35.7	7.4	20.3
*N**	10.3	14.0	1.5	20.1	1.3	31.0	34.4	32.7	30.1	17.9	9.4	44.4	32.7	39.7	20.6
A	14.2	21.5	12.8	13.7	15.5	11.0	18.9	9.4	11.5	11.4	16.9	23.3	31.9	10.5	15.0

If the exclusion criteria of 25% for 3 PPig and 15 % for TChl *a* is applied, the reference subset should include only two laboratories: *J* and *H*. However, considering that the distributed mixes were non-homogenous, the 25% exclusion criterion was not applied for

this HIP-3 exercise. The results on secondary and tertiary pigments are not discussed either because the uncertainties were already too high for the PPig.

The uncertainty range (Table 22) for HIP-3 was 14.1% for TChl *a* and 15% for the PPig. The precision (Table 23) was good for all the laboratories: 2.4% for TChl a and 3.6% for PPig. The precision results were close to those observed in HIP-1 and 2.

Table 22a. Mixed standard (mix 108) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated as a function of the method for the PPig, MVChl a and DVChl a. No reference subset was defined. For the *L* are considered only the L^+ values of 2016. Values higher than 25% are in red. The overall average for each pigment is given in the ***A*** entries.

$\|\psi\|$ mix 108	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*	PPig
D	9.0	18.6	1.7	11.1	1.2	3.2	13.3	1.0	10.3	2.7	25.6	12.7	**38.1**	13.5	2.0
L^+	1.4	**24.3**	1.7	2.8	1.9	2.2	13.3	1.0	2.6	2.7	8.9	18.0	14.4	13.8	3.9
J	14.6	**40.3**	5.2	0.0	4.9	2.2	8.2	3.8	3.8	0.7	3.4	8.5	15.6	9.5	14.8
H	7.6	18.3	12.1	2.8	19.8	8.6	22.4	5.8	9.0	0.7	11.3	**37.6**	27.1	14.7	7.5
N (all)	3.4	16.2	17.2	16.7	14.2	7.5	12.2	3.8	2.6	4.0	8.9	15.3	35.2	3.1	1.4
A	7.2	23.5	7.6	6.7	8.4	4.7	13.9	3.1	5.6	2.1	11.6	18.4	26.1	10.9	9.4

Table 23. Mixed standard (mix 108) variation coefficient ($\xi_{Pi}^{L_j}$) for the associated method corresponding to the PPig, MVChl a and DVChl a. The overall average for each pigment is given in the ***A*** entries.

ξ mix 108	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*	PPig
D	1.0	0.9	1.6	0.8	1.4	1.0	1.3	1.1	0.9	0.6	0.9	1.4	0.6	1.1	1.1
L	0.3	1.0	1.4	0.8	0.4	0.4	1.5	0.5	1.4	0.5	0.6	0.8	0.5	0.2	0.8
J	7.4	1.7	0.1	1.4	4.6	0.0	0.1	4.7	3.2	0.1	1.4	1.7	8.0	7.6	2.2
H	2.2	7.7	11.6	19.8	12.9	21.5	4.3	13.0	9.5	17.8	12.2	1.3	2.5	24.3	11.2
N (all)	1.2	1.1	2.3	3.2	0.7	12.4	1.3	3.5	1.7	1.6	1.0	2.7	1.6	1.0	2.7
A	2.4	2.5	3.4	5.2	4.0	7.1	1.7	4.6	3.3	4.1	3.2	1.6	2.6	6.8	3.6

Table 24. Mixed standard (mix 108) concentrations (mg/m^3) calculated from *J*, *L*, *D* and *N* laboratories in different dates. In L^+ are the calculation of 2016. In red HPLC results, in black spectrophotometric results (trichromatic equation applied): same letter corresponds to same vial.

mix 108 concentration (mg/m^3)	*J*	*N*	*L*	L^+	*D*
01.01.2012 (HIP-3 results)	**4.62** (average of 2 vials)	**4.6** 1 vial **6.73** average of 2 vials	**3.88** average of 3 vials	**5.61**	**6.03**
30.05.2013	**4.17a**				
03.06.2013	**4.89b**				
27.06.2013		**4.95** 1 vial			
05.09.2013					**6.33(a, b)**
01.10.2013	**6.14c** **6.16d**				
07.10.2013			**6.15c / 7.865c** **6.22d / 7.92d** **6.16e / 7.77e**	**5.56c** **5.61d** **5.51e**	

In 2016, *L* resubmitted the mixed standard calculation, finding out a problem in the calculation done for the first data submission. The new values, indicated by the entries L^+ in Table 21, were much closer to the values measured from all the other laboratories. In Table 22a laboratories are compared with the new values of *L*: in this case the uncertainties among laboratories were 7.2 % for TChl *a* and 9.4% for the PPig: double respect what obtained in the previous HIPs. Even with this new computation, no subset was defined, but because the uncertainties of all the laboratories were within the fixed threshold of 25% for 3 PPig and 15% TChl *a.*

8.2.4 HIP-4 results for mixed standards

Two different pigment concentration ranges of mixed standards were distributed during the HIP-4 exercise: mix-1 112 with a TChl *a* concentration (measured by spectrophotometry and certified by DHI) between 3-6 mg/m^3 and mix-2 102 with a certified TChl *a* concentration of 0.1-0.2 mg/m^3.

For mix-1 112*, D* provided 14 analyses of the standard, *L* 18*, J* 7, *E* 12 and *N* 9. For the mix-2 102 (low range of concentration) *N* did not submit results, *D* submitted 2 measurements and, *J, E*, and *L* 3 measurements each.

The mix-1 112 standard had to be analysed in two different injection sequences in order to assess the method stability with time and between mobile phase changes and to analyse both the mixes at least three times.

According to the laboratory subset reference, *E* had to be excluded due to high differences (higher than 25%) with respect to the laboratory average for five PPig both in mix-1 112 (TChl *a*, Caro, HexFuco, Per and Zeax) and mix-2 102 (all PPig). Note that *L* laboratory is the only one with a TChl *a* uncertainty within 5% for the mix-1 112 and mix-2 102.

Table 25. Mixed standard (mix-1 112) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated as a function of the method for the PPig, MVChl *a* and DVChl *a*. In red, values higher than 25% (15% for the TChl *a*). The overall average for each pigment for the reference subset is given in the $\boldsymbol{A^s}$ entries.

$\lvert\psi\rvert$ mix-1 112	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*	PPig
D	14.1	11.5	1.0	10.6	2.1	22.6	5.3	1.0	3.1	0.3	1.4	7.7	8.0	15.4	7.4
L	5.4	7.9	12.1	3.3	1.0	18.3	0.9	6.4	1.6	0.2	9.4	9.2	12.9	7.9	6.9
J	12.1	14.1	15.7	0.2	3.4	18.7	3.9	3.3	2.1	1.5	1.3	13.0	5.0	12.4	7.6
N	7.3	5.3	2.6	7.1	4.5	59.7	10.1	4.0	6.8	1.7	6.8	11.5	26.0	10.9	11.7
E	>100	17.2	24.2	>100	7.0	>100	14.3	7.5	1.6	8.1	>100	>100	21.0	70.8	41.2
$\boldsymbol{A^s}$	9.7	9.7	7.9	5.3	2.7	29.8	5.1	3.7	3.4	0.9	4.7	10.3	13.0	11.7	8.4

Table 26. Mixed standard (mix-1 112) percent error ($\xi_{Pi}^{L_j}$) for the associated method corresponding to the PPig, MVChl *a* and DVChl *a*. The overall average for each pigment for the reference subset is given in the $\boldsymbol{A^s}$ entries.

ξ mix-1 112	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*	PPig
D	0.8	0.9	0.8	1.1	1.4	2.0	0.5	1.9	2.1	1.0	0.6	1.6	2.7	0.8	1.3
L	0.7	0.7	0.4	0.7	0.6	0.4	1.0	0.7	1.6	0.4	0.4	0.5	0.8	0.8	0.7
J	1.1	5.5	4.5	4.5	0.9	0.9	0.8	0.8	4.0	1.0	5.5	2.1	3.7	1.2	2.6
N	2.7	2.3	2.7	2.5	3.7	3.4	3.3	3.1	5.8	7.1	3.9	5.1	3.5	2.8	3.7
E	15.9	8.3	1.5	..	3.1	2.0	2.2	1.9	5.8	5.4	2.7	3.3	14.3	16.5	6.4
$\boldsymbol{A^s}$	1.3	2.3	2.1	2.2	1.7	1.6	1.4	1.6	3.4	2.4	2.6	2.4	2.7	1.4	2.1

The reference subset for HIP-4 was composed of four laboratories *J, D, L* and *N*, implementing methods based on the Van Heukelem & Thomas method. The uncertainty within the reference subset was 8.4% for the PPig, and 9.7% for the TChl *a* (Table 25). While the uncertainty worsened with respect to HIP-1 and 2, the subset precision remained at a good level: 1.3% for the TChl *a* and 2.1% for PPig (Table 26).

The results for the secondary and tertiary pigments are reported for three laboratories. *N* only reported DVChl *a* and MVChl *a*, and is included in the subset for these two pigments. The quantified pigments were: Chlide *a*, MVChl *a*, DVChl *a*, Neo, Pras, Viol and Lut. (Table 27 and 28). For the MVChl *a*, the results in terms of uncertainty (11.7%) and precision (1.4%) were similar to those obtained for the PPig. This was also the case for the others, with the exception of Chlide *a*, that showed a very poor uncertainty (53.4%) and precision (16%).

For mix-2 102, the results were similar to those obtained for the higher range of concentration mix, confirming that the methods were working well along the full concentration range of natural samples analysed. The uncertainty for the PPig was 5.2% and 8.2% for the TChl *a* (Table 29). The precision was respectively 1.3% for the TChl *a* and 1.6% for the PPig.

The results for the secondary and tertiary pigments are reported for three laboratories. The pigments quantified were: Chlide *a*, MVChl *a*, DVChl *a*, Neo, Pras, Viol, Lut (Table 31 and 32). The MVChl *a* results, in terms of uncertainty (9.0%) and precision (1.3%), were close to those obtained for the PPig.

Table 27. Mixed standard (mix-1 112) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated as a function of the method for the secondary and tertiary pigments. The overall average for each pigment is given in the ***A*** entries.

$\|\psi\|$ mix-1 112	Chlide *a*	DVChl *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	80.1	8.0	15.4	12.9	1.5	0.3	11.9
L	44.1	12.9	7.9	11.1	0.9	3.0	12.5
J	36.0	5.0	12.4	1.8	2.4	3.3	0.6
N	- -	26.0	10.9	- -	- -	- -	- -
A	53.4	13.0	11.7	8.6	1.6	2.2	8.3

Table 28. Mixed standard (mix-1 112) variation coefficient ($\xi_{Pi}^{L_j}$) associated to each method corresponding to secondary and tertiary pigments. The overall average for each pigment is given in the ***A*** entries.

ξ mix-1 112	Chlide *a*	DVChl *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	1.8	2.7	0.8	0.8	0.6	4.9	3.3
L	1.4	0.8	0.8	0.5	0.4	0.7	0.6
J	45.7	3.7	1.2	1.5	0.9	1.4	3.3
N	- -	3.5	2.8	- -	- -	- -	- -
A	16.3	2.7	1.4	0.9	0.6	2.3	2.4

Table 29. Mixed standard (mix-2 102) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated as a function of the method for the PPig, MVChl *a* and DVChl *a*. In red, values higher than 25% (15% for the TChl *a*). The overall average of each pigment for the reference subset is given in the $\boldsymbol{A^s}$ entries.

$\|\psi\|$ mix-2 102	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*	PPig
D	9.4	9.4	8.0	3.6	4.1	2.7	2.7	1.1	3.2	0.7	2.2	5.1	0.8	10.2	4.3
L	3.0	5.4	9.7	3.7	3.7	1.0	5.3	1.6	5.6	2.4	8.1	13.7	1.8	3.4	5.3
J	12.4	14.8	17.7	0.1	0.3	1.7	2.6	2.7	2.4	1.7	5.9	8.6	2.6	13.5	5.9
E	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
$\boldsymbol{A^s}$	8.2	9.9	11.8	2.5	2.7	1.8	3.5	1.8	3.7	1.6	5.4	9.2	1.7	9.0	5.2

Table 30. Mix standard (mix-2 102) variation coefficient ($\xi_{Pi}^{L_j}$) for the associated method corresponding to PPig, MVChl *a* and DVChl *a*. The overall average for each pigment for the reference subset is given in the $\boldsymbol{A^s}$ entries.

ξ mix-2 102	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	DVChl *a*	MVChl *a*	PPig
D	1.6	0.6	0.8	1.0	0.8	0.1	0.1	0.9	0.1	0.5	0.1	0.9	1.5	1.6	0.6
L	0.8	0.5	5.3	0.9	0.7	1.2	0.5	1.2	0.8	0.0	1.2	1.4	2.1	1.0	1.2
J	1.6	7.6	1.2	1.2	2.4	2.3	2.0	3.1	1.7	3.3	9.4	0.8	2.3	1.6	3.1
E	1.1	1.8	2.4	- -	2.1	0.9	6.2	0.9	3.9	3.0	2.1	4.1	1.8	1.2	2.6
$\boldsymbol{A^s}$	1.3	2.9	2.4	1.1	1.3	1.2	0.9	1.8	0.9	1.3	3.5	1.1	1.9	1.4	1.6

Table 31. Mixed standard (mix-2 102) absolute percent difference ($|\psi|_{Pi}^{L_j}$) evaluated as a function of the method for secondary and tertiary pigments. *J* only reported the LOD for Neo and Viol. The overall average for each pigment is given in the $\boldsymbol{A}$ entries.

$\|\psi\|$ mix-2 102	DVChl *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	0.8	10.2	58.8	2.6	55.8	12.6
L	1.8	3.4	41.2	0.5	44.2	13.0
J	2.6	13.5	(LOD)	3.1	(LOD)	0.4
$\boldsymbol{A}$	1.7	9.0	50.0	2.1	50.0	8.7

Table 32. Mixed standard (mix-2 102) variation coefficient ($\xi_{Pi}^{L_j}$) associated to each method corresponding to secondary and tertiary pigments. The overall average of each pigment is given in the $\boldsymbol{A}$ entries.

ξ mix-2 102	DVChl *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	1.5	1.6	0.6	0.8	6.4	0.4
L	2.1	1.0	1.0	0.5	1.2	4.3
J	2.3	1.6	- -	2.4	- -	1.9
$\boldsymbol{A}$	1.9	1.4	0.8	1.3	3.8	2.2

8.3 Method Validation: analysis on natural samples

The statistical approach used for the validation of methods on natural samples, is the same as that described in Chapter 8.2 for the evaluation of mixed standards, with the introduction of the replicates (not present for the mixed standards).

For each single pigment concentration ($\tilde{C}_{Pi}^{L_j}$) of a sample, analysed by each laboratory (L_j), the average concentration ($\overline{C}_{Pi}^{L_j}$) is calculated for the batch of triplicates analysed by each laboratory. N_R is the number of replicates and S_k is the sampling station:

3) $$\overline{C}_{Pi}^{L_j}(S_k) = \frac{1}{N_R}\sum_1^{N_R} \tilde{C}_{Pi}^{L_j}(S_k)$$

The standard deviation ($\sigma_{Pi}^{L_j}$) and the percent variation coefficient ($\xi_{Pi}^{L_j}$) are calculated as:

4) $$\sigma_{Pi}^{L_j}(S_k) = \sqrt{\frac{1}{N^{L_j}}\sum_1^{N^{L_j}} (\tilde{C}_{Pi}^{L_j}(S_k) - \overline{C}_{Pi}^{L_j}(S_k))}$$

6) $$\xi_{Pi}^{L_j}(S_k) = 100\frac{\sigma_{Pi}^{L_j}(S_k)}{\overline{C}_{Pi}^{L_j}(S_k)}$$

The pigment average ($\overline{C}_{Pi}^{A}$) for each batch across the laboratories identified as part of the subset defined for the mixed standard comparison (A) is calculated as:

6) $$\overline{C}_{Pi}^{A}(S_k) = \frac{1}{N_L}\sum_1^{N_L} \tilde{C}_{Pi}^{A}(S_k)$$

N_L is the number of laboratories quantifying the pigment in the reference subset.

The absolute unbiased percent difference, $|\psi|_{Pi}^{L_j}$, is calculated for each pigment of a single laboratory with respect to the average value across the subset laboratories .

7) $$|\psi|_{Pi}^{L_j}(S_k) = 100\left|\frac{\tilde{C}_{Pi}^{L_j}(S_k) - \overline{C}_{Pi}^{A}(S_k)}{\overline{C}_{Pi}^{A}(S_k)}\right|$$

The difference between a given laboratory and the subset average must not exceed 25% for more than 3 PPig (15 % for TChl *a*) for this laboratory to be included in the subset. *C* was excluded from the average subset for the HIP-2 exercise due to differences higher than 25 % for 4 pigments: TChl *b*, But-fuco, Perid and Zeax.

8.3.1 HIP-1 results of natural samples

The HIP-1 exercise was initially organized for 12 batches of triplicates comprising 10 batches of 25 mm diameter samples and 2 batches of 47 mm diameter samples. The two 47 mm batches are included because, until 2009, *J* collected and routinely analysed 47 mm filter size HPLC samples.

For the 10 batches of 25 mm filters a good agreement was generally observed among the three laboratories. The two batches of 47 samples led to differences in pigment quantification. This can probably be attributed to the lower frequency of routine analysis with 47 mm filters size for two of the participant laboratories (*D* and *L*).

L laboratory encountered a mobile phase contamination problem during the HIP-1 analysis (described in Annex I). A few extra batches of replicates were delivered to *L* for further analysis, however the results have not been taken into account in this report.

8.3.1.1 Comparison on 25 mm batches

The laboratories involved in this exercise presented major differences in sample extraction procedures (see Table 4).

As there is a good agreement on the mixed standard analyses (Chapter 8.2.1) between the three participants, the averages of natural samples for all three laboratories (Table 33) were considered as true values for the computation of the $\xi_{Pi}^{L_j}$ and $|\psi|_{Pi}^{L_j}$ between the laboratories (Table 34 and 35).

None of the laboratories exceeded the 25% difference for more than 3 PPig, confirming the good agreement between the three laboratories on natural samples too.

The average uncertainties for TChl *a*, increase from 3.4% for the mix 106 pigments standards (Table 13) to 7.8% for the natural samples (Table 34). The PPig average uncertainties are 12 %, increased with respect to the 6.7% obtained for the mix 106 standards. At the same time the average precision in the measurements rose to 7.2% for the PPig (Table 35). The 4 % difference can be attributed to intra replicate variability.

Table 33. HIP-1 PPig and MVChl *a* concentration average values (mg/m^3) for the replicates series. The overall average of each pigment is given in the $\overline{C}_{Pi}^{A}$ entries.

SAMPLE BATCH	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*
G	0.922	0.108	0.156	0.037	0.061	0.309	0.031	0.077	0.009	0.086	0.007	0.06	0.912
H	2.301	0.074	0.391	0.06	0.016	0.091	0.038	0.161	0.036	0.73	0.027	0.021	1.658
I	1.006	0.039	0.168	0.03	0.024	0.119	0.043	0.099	0.018	0.283	0.012	0.025	0.871
L	2.215	0.04	0.435	0.059	0.013	0.084	0.035	0.246	0.036	0.669	0.031	0.016	1.26
M	0.400	0.033	0.067	0.014	0.041	0.111	0.012	0.018	0.003	0.046	0.004	0.027	0.393
N	0.797	0.028	0.158	0.025	0.021	0.095	0.031	0.096	0.013	0.252	0.011	0.021	0.624
P	0.797	0.025	0.161	0.022	0.021	0.081	0.029	0.071	0.005	0.244	0.01	0.016	0.66
Q	1.469	0.046	0.22	0.044	0.011	0.058	0.05	0.152	0.023	0.361	0.034	0.016	0.995
V	2.505	0.174	0.505	0.091	0.094	0.758	0.186	0.169	0.026	0.46	0.028	0.071	2.594
Z	2.059	0.136	0.352	0.069	0.067	0.537	0.13	0.139	0.021	0.325	0.018	0.06	1.858
max	2.505	0.174	0.505	0.091	0.094	0.758	0.186	0.246	0.036	0.73	0.034	0.071	2.594
$\overline{C}_{Pi}^{A}$	1.447	0.07	0.261	0.045	0.037	0.224	0.058	0.123	0.019	0.345	0.018	0.033	1.182
min	0.404	0.025	0.067	0.014	0.011	0.058	0.012	0.018	0.003	0.046	0.004	0.016	0.393

Table 34. Absolute percent difference ($|\psi|_{Pi}^{L_j}$) for natural samples as a function of the method for PPig and MVChl *a*. The overall average for each pigment is given in the ***A*** entries.

$\|\psi\|$	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	3.1	5.8	14.9	19.6	5.5	7.3	8.7	7.8	13.5	8.0	16.4	15.3	6.5	10.1
L	9.1	16.0	5.6	15.3	11.0	5.8	5.6	11.1	13.0	6.1	13.4	9.0	4.6	10.5
J	11.2	15.4	17.4	14.7	13.6	12.8	14.3	18.1	24.9	13.8	11.4	17.4	9.3	15.4
A	7.8	12.4	12.7	16.5	10.0	8.6	9.5	12.3	17.2	9.3	13.7	13.9	6.8	12

The secondary and tertiary pigments reported from all three laboratories are: Chlide *a*, MVChl *a* and Neo, Pras, Viol and Lut. *D* did not report DVChl *a* for natural samples, but only for mix 106. The uncertainty and the precision obtained for the secondary and tertiary pigments are higher than the values obtained for the PPig with the exception of MVChl *a* (uncertainty 6.8% and precision 8%).

Table 35. Percent variation coefficient ($\xi_{Pi}^{L_j}$) for natural samples as a function of the method for PPig and MVChl *a*. The overall average for each pigment is given in the ***A*** entries.

ξ	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	4.2	4.6	6.5	7.8	4.4	3.7	4.7	7.7	12.5	6.3	7.3	3.1	7.5	6.1
L	5.5	5.2	3.0	5.9	4.1	2.6	4.5	5.3	8.4	6.5	14	4.9	8.0	5.8
J	7.8	8.0	7.5	9.3	7.5	6.8	8.7	11.1	17.2	9.0	11.2	14	8.4	9.8
A	5.8	5.9	5.7	7.7	5.3	4.4	6.0	8.0	12.7	7.3	10.8	7.3	8.0	7.2

Table 36. Absolute percent difference ($|\psi|_{Pi}^{L_j}$) for natural samples as a function of the method for secondary pigments. *D* did not report DVChl *a* for natural samples (only for mix 106). The overall average for each pigment is given in the ***A*** entries.

$\|\psi\|$	Chlide *a*	MVChl *a*	Pheo *a*	Neo	Prasi	Viola	Lut
D	32.4	6.5	14.1	4.6	12.8	7.8	22.3
L	37.6	4.6	35.7	15.4	9.0	21.0	30.7
J	20.1	9.3	25.3	17.7	17.0	22.0	19.1
A	30.0	6.8	25.0	12.5	13.0	16.9	24.1

Table 37. Percent variation coefficient ($\xi_{Pi}^{L_j}$) for natural samples as a function of the method for secondary pigment. The overall average of each pigment is given in the ***A*** entries.

ξ	Chlide *a*	MVChl *a*	Pheo *a*	Neo	Prasi	Viola	Lut
D	6.0	7.5	0.0	6.9	8.5	5.9	10.1
L	3.1	8.0	0.0	11.4	7.3	6.4	6.3
J	7.4	8.4	14.8	15.7	21.1	8.6	7.5
A	5.5	8.0	4.9	11.3	12.3	7.0	8.0

8.3.1.2 Comparison on 47 mm batches

The two series of 47 mm triplicates (Table 3) distributed for HIP-1 have been compared using the same statistic approach used for the 25 mm but considering the relative percent difference (ψ) instead of the absolute ($|\psi|$). The average of the three laboratories results is considered to be the true value.

The comparison (Table 38) evidences an overestimation of *D* and a systematic under estimation of *L*. These results are in contradiction with what emerged from the 25 mm series comparison, where the three laboratories showed a generally good agreement and no specific trend. Therefore, these two 47 mm filter size series were not considered together with the 25 mm for the laboratory evaluation, as initially planned. By taking into account these results a specific test has been proposed, based on filter size on a larger

and statistical relevant number of samples, for the following HIP-2 intercalibration exercise.

Table 38. Average concentration (in mg/m^3) for the two 47 mm replicates series C and Y, and relative percent difference ($\Psi_{Pi}^{L_j}$) of all 47 mm replicates for each method.

	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax
$\overline{C}_{Pi}(C)$ (mg/m3)	0.405	0.015	0.103	0.015	0.009	0.037	0.016	0.067	0.007	0.193	0.012	0.007
$\overline{C}_{Pi}(Y)$ (mg/m3)	0.445	0.040	0.082	0.011	0.013	0.062	0.017	0.032	0.003	0.151	0.008	0.003
Ψ_{Pi}^{J}	-11.4	-13.2	-10.2	12.1	-16.2	-15.0	-11.0	-5.8	7.7	-14.0	-17.6	4.0
Ψ_{Pi}^{D}	44.0	57.5	42.2	53.4	50.0	45.0	48.5	48.4	28.2	42.1	15.2	50.0
Ψ_{Pi}^{L}	-32.6	-44.3	-32.0	-65.5	-33.8	-29.9	-37.5	-42.7	-35.9	-28.1	2.4	-54.0

8.3.2 HIP-2 results of natural samples

The HIP-2 exercise was divided in two parts. A general part involving all the laboratories and focused on the method comparison based on 25 mm filters triplicates. A second part involving only the three laboratories of HIP-1 exercise (*D*, *L* and *J*) also analysed 47 mm filter batches.

This side-exercise on 47 mm filters is limited to laboratories that already demonstrated a good agreement during HIP-1. The aim of this extraction exercise was to test the equivalence of the extraction method when applied to different filter sizes. The HIP-2 discussion is divided into two parts: the general comparison of 25 mm filters and the side-exercise on 47 mm.

Subsequently to the HIP-2 results presentation (MERIS Validation Team meeting, 8-10th March 2011, Ispra), *N* asked to resubmit its results. These new results are discussed in the following.

8.3.2.1 Comparison on 25 mm batches

The laboratory uncertainties ($|\psi|_{Pi}^{L_j}$) are initially evaluated with respect to the average of the reference laboratory subset defined during the mixed standard validation step including *D, J, C* and *L*. The AM batch was excluded from the statistical calculation because of high variability evidenced across all laboratories.

Following the criterion of excluding from the reference subset the laboratories that exceed 25% difference for more than 3 PPig, the reference subset was redefined with the exclusion of the *C* laboratory. *C* was penalized due to high amounts of non-quantified (below LOD) pigments. The *C* LOD values were much higher than those reported by other laboratories. As the *C* method requires a low sample injection volume (50 µL), this choice could explain the high values of non-quantified pigments and LOD values. As established in Chapter 5, the effective LOD is used instead of the zero value for the non-quantified pigments (Table 39). The average of the three laboratories *D*, *L* and *J* ($\overline{C}_{Pi}^{A^s}$ in Table 39) was considered as the true value for the computation of $\xi_{Pi}^{L_j}$ and $|\psi|_{Pi}^{L_j}$.

Table 39. HIP-2 PPig and MVChl *a* concentration average values (in mg/m^3) for the replicates series. The values are calculated for the reference subset (*D, L* and *J*). The overall average of each pigment for the reference subset is given in the $\overline{C}_{Pi}^{A^s}$ entries.

SAMPLE BATCH	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*
B	1.207	0.046	0.212	0.075	0.011	0.104	0.018	0.153	0.032	0.431	0.060	0.122	1.083
D	3.886	0.109	0.784	0.135	0.018	0.072	0.037	0.129	0.029	1.937	0.055	0.128	3.436
G	1.046	0.042	0.181	0.064	0.013	0.107	0.022	0.118	0.021	0.319	0.037	0.152	0.955
I	3.211	0.130	0.662	0.097	0.024	0.075	0.039	0.106	0.021	1.563	0.083	0.091	2.972
T	0.107	0.007	0.014	0.009	0.007	0.024	0.001	0.012	0.002	0.007	0.006	0.044	0.095
V	0.107	0.006	0.022	0.007	0.013	0.037	0.001	0.009	0.001	0.010	0.003	0.017	0.106
Z	0.081	0.006	0.016	0.005	0.010	0.027	0.000	0.011	0.001	0.007	0.002	0.015	0.080
AA	0.088	0.004	0.018	0.009	0.009	0.031	0.000	0.011	0.001	0.009	0.003	0.017	0.087
AF	2.151	0.234	0.275	0.141	0.026	0.190	0.078	0.127	0.023	0.538	0.059	0.171	2.093
AH	0.185	0.011	0.034	0.042	0.013	0.042	0.002	0.015	0.001	0.042	0.003	0.023	0.182
AI	0.975	0.098	0.165	0.056	0.033	0.215	0.027	0.102	0.017	0.185	0.018	0.126	0.950
AM	0.358	0.032	0.064	0.014	0.018	0.092	0.011	0.042	0.007	0.075	0.009	0.055	0.351
max	3.886	0.234	0.784	0.141	0.033	0.215	0.078	0.153	0.032	1.937	0.083	0.171	3.436
$\overline{C}_{Pi}^{A^s}$	1.330	0.074	0.249	0.061	0.018	0.095	0.024	0.076	0.014	0.543	0.032	0.087	1.217
min	0.081	0.004	0.014	0.005	0.007	0.024	0.000	0.009	0.001	0.007	0.002	0.015	0.080

The average TChl *a* uncertainties for the subset increase from 3.5 %, as obtained with the mix 107 standard, to 4.5% for natural samples (Table 40), while for the PPig, the uncertainty ranges from 4.5% (mix 107) to 11.4% (natural samples).

The subset PPig precision decreases from 6.8% of mix 107 to 12.2% for natural samples (Table 41). The 4% variability can be related to the lack of homogeneity between replicates, similarly to what observed during the HIP-1 exercise.

N asked to resubmit its data after the first presentation of the comparison results (MERIS Validation Team meeting, 8-10th March 2011, Ispra) by recalculating the data with a different approach. Before the resubmission, *N* uncertainties for TChl *a* were 15%, while after the resubmission, the uncertainties rose to 21.3% for TChl *a*, and from 42.2% to the 46.2% for the PPig.

Table 40. Absolute percent difference ($|\psi|_{Pi}^{L_j}$) for natural samples as a function of the method for PPig and MVChl a. *N'* are the *N* resubmitted data. Red values higher than 25% (15% for the TChl *a*). The overall average for each pigment for the reference subset is given in the $\boldsymbol{A^s}$ entries.

$\lvert\psi\rvert$	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	3.9	10.8	5.8	14.2	2.3	5.1	9.4	4.1	15.6	3.5	22.7	12.7	5.7	9.2
L	5.0	11.3	7.2	32.0	9.5	5.4	11.6	5.4	8.3	6.3	10.6	31.0	7.8	12.0
J	4.6	7.1	10.9	26.3	9.7	10.1	20.0	5.2	16.0	8.9	17.0	20.2	4.6	13.0
C	23.8	>100	27.3	82.1	>100	25.2	35.3	28.9	26.8	19.8	36.2	41.3	26.0	40.7
N	15.1	17.3	78.0	39.6	31.8	20.4	88.1	43.6	56.7	28.4	>100	45.5	--	42.2
N'	21.3	10.3	90.9	40.6	31.2	16.7	86.0	39.1	55.2	23.6	>100	39.5	--	46.2
$\boldsymbol{A^s}$	4.5	9.7	8.0	24.1	7.2	6.9	13.7	4.9	13.3	6.2	16.8	21.3	6.0	11.4

The secondary and tertiary pigments, as for the mix 107, were only compared for three laboratories: *J*, *D* and *L.* The pigments reported are Chlide *a*, MVChl *a* and Neo, Pras, Viol and Lut. Although *C* reported MVChl *a* and the DVChl *a,* it was not included in the quality

subset. As already observed for the mix standard 107, the uncertainty and the precision for the secondary and tertiary pigments were poorer than those obtained for the PPig, with the exception of MVChl *a*. Chlide *a* was worse compared to what obtained for the mix 107 too: the uncertainty rose from 6.7% of the mix 107 (Table 17) to 40.6% for the natural samples (Table 42) and the precision decreased to 22.8% (Table 43) for the natural sample (previously 0.7% for the mix 107, Table 18).

Table 41. Percent variation coefficient ($\xi_{Pi}^{L_j}$) for natural samples as a function of the method for PPig and MVChl *a*. The overall average for each pigment for the reference subset is given in the $\boldsymbol{A^s}$ entries.

ξ	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	9.1	9.3	11.0	9.4	10.9	8.9	43.1	10.5	10.6	12.7	15.8	8.1	10.6	13.3
L	6.2	7.1	6.9	10.4	6.1	4.9	9.4	7.3	13.3	8.0	12.4	7.4	6.2	8.3
J	9.2	19.4	9.6	15.8	7.8	7.6	28.7	11.0	21.9	12.0	22.3	15.7	9.7	15.1
C	6.4	0.9	1.7	3.0	0.0	1.3	2.7	4.0	9.1	4.9	2.9	2.4	6.7	3.3
N	6.7	20.0	11.3	11.7	7.5	6.2	5.3	8.3	6.0	13.5	2.0	13.0	--	9.3
$\boldsymbol{A^s}$	8.2	12.0	9.2	11.8	8.3	7.2	27.1	9.6	15.3	10.9	16.9	10.4	8.8	12.2

Table 42. Absolute percent difference ($|\psi|_{Pi}^{L_j}$) for natural samples as a function of the method for secondary and tertiary pigments For natural samples *D* did not report the DVChl *a* (but only for the mix 107) and for the Pras reported only the LOD.

$\lvert\psi\rvert$	Chlide *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	47.8	5.7	3.5	53.7	12.6	60.4
L	33.3	7.8	6.3	19.5	13.6	63.1
J	26.5	4.6	8.9	43.2	12.2	51.1
C	- -	26.0	- -	- -	- -	- -
$\boldsymbol{A^s}$	40.6	6.0	4.9	36.6	13.1	61.7

Table 43. Percent variation coefficient ($\xi_{Pi}^{L_j}$) for natural samples as a function of the method for secondary and tertiary pigments. For natural samples *D* did not report the DVChl *a* (but only for the mix 107) and for the Pras reported only the LOD.

ξ	Chlide *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	31.4	10.6	14.5	(LOD)	12.7	57.5
L	15.3	6.2	9.9	7.6	7.1	12.9
J	21.7	9.7	22.0	10.7	16.7	53.1
C	- -	6.7	- -	- -	- -	- -
$\boldsymbol{A^s}$	22.8	8.8	15.5	9.1	12.2	41.2

8.3.2.2 Comparison of 47 mm batches (only *D*, *L* and *J*)

The secondary exercise involved 3 laboratories (*J*, *D* and *L*) that routinely analyse or have previously analysed 47 mm filter size samples. Uncertainties between *J, D* and *L* over the 12 batches of 25 mm size filters were lower than 13% (see Table 41) and similar differences were expected for the 47 mm size batches.

For each laboratory the difference with respect to the filter size was evaluated within the same batch (Table 44).

Table 44. Relative percent difference ($\Psi_{Pi}^{L_j}$) for natural samples as a function of the filters size for PPig and MVChl a.

	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*
D	2.4	13.0	3.2	6.9	-2.5	-0.6	1.8	3.8	0.3	6.4	-4.4	1.1	15.9
L	40.2	36.2	26.7	54.4	26.5	22.6	24.0	24.5	23.3	23.8	11.8	48.2	41.6
J	8.4	6.5	22.8	15.4	-0.7	3.9	4.2	5.6	-2.4	7.6	14.0	-1.9	10.9

J and *D* uncertainties with respect to the filter size were comparable (lower than 15%) to the results obtained within the 25 mm series. *L* systematically obtained higher values for the 47 mm filters, obtaining on average a 40.2 % difference for TChl *a*. The 25 mm pigment concentrations were therefore underestimated with respect to the 47 mm filters.

For the 47mm filters, the amount of extraction solvent used by *L* was higher than that used by *D* and *J* (8 mL vs 5 mL respectively; see Table 7) and this could explain the systematic difference obtained.

The *L* laboratory was excluded from the subset average in the evaluation of the uncertainties between the 25 mm and the 47 mm filter size samples.

In table 45, the percent difference between 25 mm and 47 mm filters of quantified pigments for *D* and *J* is presented. The uncertainties linked to the filter size are 6.5 % for TChl *a* and 9.2 % for PPig.

Table 45. Absolute percent difference ($|\psi|_{Pi}^{L_j}$) for natural samples as a function of the filter size for PPig and MVChl a. The overall average for each pigment is given in the ***A*** entries.

Series compared	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
B/C	8.7	1.7	9.0	25.1	20.1	2.5	16.8	4.1	11.3	4.3	30.5	5.6	18.6	11.6
D/E	11.0	12.3	13.9	13.3	7.9	2.9	7.2	7.1	3.2	9.7	10.2	9.4	20.6	9.0
G/H	1.3	11.8	4.8	7.4	0.2	1.9	3.7	1.6	1.8	0.5	6.8	14.8	8.2	4.7
I/L	12.2	12.6	18.3	8.8	1.1	6.3	10.4	10.4	18.5	13.5	4.9	6.2	19.2	10.3
AF/AG	2.4	7.2	12.2	0.2	12.0	4.4	4.7	7.8	7.6	6.5	11.5	3.5	4.7	6.7
AI/AL	3.4	14.6	24.2	31.0	5.6	7.7	18.3	8.8	10.1	9.4	15.8	5.9	9.5	12.9
A	6.5	10.0	13.7	14.3	7.8	4.3	10.2	6.6	8.8	7.3	13.3	7.6	13.5	9.2

8.3.3 HIP-3 results of natural samples

The HIP-3 intercomparison extended the same extraction exercise on different filter sizes as already proposed in HIP-2, to all the participants. Indeed, the extraction exercise for 47 mm filters is not discussed in the present report, due the high differences that already emerged from comparison of the mix 108 standard and 25 mm natural samples results.

Here only the comparison between the 25 mm natural samples is discussed (Table 46).

Due to the analysis of mix standard, no reference subset was defined. This is also applied in the case of the *L* recalculated values. After comparing the data for natural samples, the only possible quality subset that could be defined should consist of *D*, that had only one pigment higher than 25% with respect to the average and *L* that had two PPig (Zeax and TChl *c*) higher of 25% and one PPig, Caro, on the limit of 24.8% (Table 47). Due to doubts concerning the homogeneity of the distributed mixes standard, it was decided to not establish any reference subset even for natural samples and to compare all the laboratories together.

Table 46. HIP-3 PPig and MVChl *a* concentration average values (in mg/m^3) for the replicates series. The overall average of each pigment is given in the $\overline{C}_{Pi}^{\,A}$ entries.

SAMPLE BATCH	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*
A	0.616	0.027	0.133	0.015	0.010	0.097	0.004	0.041	0.005	0.234	0.012	0.003	0.562
B	0.913	0.017	0.266	0.021	0.005	0.039	0.008	0.081	0.007	0.449	0.023	0.003	0.792
C	0.322	0.020	0.071	0.011	0.005	0.025	0.001	0.047	0.005	0.124	0.020	0.003	0.287
D	4.285	0.070	0.658	0.129	0.021	0.228	0.065	0.583	0.073	1.860	0.156	0.021	3.776
F (no *N*)	0.328	0.023	0.037	0.020	0.007	0.061	0.029	0.051	0.007	0.064	0.024	0.017	0.324
H	0.266	0.025	0.030	0.017	0.005	0.060	0.013	0.054	0.008	0.039	0.026	0.020	0.258
L	0.172	0.016	0.017	0.012	0.008	0.047	0.004	0.036	0.006	0.022	0.011	0.012	0.166
N	0.140	0.017	0.012	0.011	0.004	0.031	0.002	0.025	0.004	0.016	0.008	0.013	0.137
Q	0.153	0.030	0.018	0.013	0.004	0.036	0.001	0.023	0.003	0.015	0.008	0.017	0.149
R	4.153	0.040	0.546	0.147	0.025	0.087	0.068	0.821	0.078	1.999	0.092	0.050	3.615
S	0.111	0.014	0.012	0.017	0.002	0.021	0.001	0.018	0.003	0.021	0.007	0.008	0.108
W (no *J*)	0.230	0.006	0.054	0.011	0.010	0.033	0.004	0.048	0.004	0.137	0.006	0.014	0.210
max	4.285	0.070	0.658	0.147	0.025	0.228	0.068	0.821	0.078	1.999	0.156	0.050	3.776
$\overline{C}_{Pi}^{\,A}$	0.974	0.025	0.154	0.035	0.009	0.064	0.017	0.155	0.017	0.415	0.033	0.015	0.865
min	0.111	0.006	0.012	0.011	0.002	0.021	0.001	0.018	0.003	0.015	0.006	0.003	0.108

Table 47. Absolute percent difference ($|\psi|_{Pi}^{L_j}$) for natural samples as a function of the method for PPig and MVChl *a*. In red, values higher than 25% (15% for the TChl *a*). The overall average for each pigment is given in the ***A*** entries.

$\|\psi\|$	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	12.3	11.4	18.4	29.1	15.4	12.2	13.8	16.1	12.0	16.4	13.8	22.5	12.7	19.5
L	8.5	11.6	25.0	24.8	15.5	10.9	17.1	15.8	12.2	13.8	12.2	51.2	11.7	25.7
J	24.4	13.1	28.5	27.7	20.0	36.0	29.8	41.0	32.2	43.6	23.5	36.9	25.2	33.6
H	20.4	3.8	52.2	48.8	- -	54.2	25.7	45.4	28.2	52.4	- -	52.6	22.2	37.0
N	25.8	8.8	29.8	32.9	38.4	19.1	41.2	26.4	31.2	21.9	26.0	36.2	30.1	26.4
A	18.3	9.7	30.8	32.7	17.8	26.5	25.5	28.9	23.2	29.6	15.1	39.9	20.4	24.5

The TChl *a* uncertainty for natural samples is 18.3% on average (Table 47). This is significantly worse compared to what was obtained during HIP-1 (7.7%) and HIP-2 (4.5%). Similar results have been observed for the PPig that have an uncertainty of 24.5% while it was 11.9% and 11.4% respectively in HIP-1 and 2. Considering that a homogenous reference subset could not be established, the results of all laboratories were consequently affected. If HIP-2 TChl *a* uncertainty results on natural samples for *D*, *J*, *L* and *N* were to be compared with those obtained during HIP-3, *D* would change from 3.9% to 12.3%, *L* from 5% to 8.5%, *J* from 4.6% to 24.4% and *N*, who was not among the HIP-2 reference subset, from 15.1% to 25.8% (Table 40 and 47). Results with the *D*-*L* subset are shown

in Table 47a: in this scenario *J* improves its uncertainty from 24.4 % to 16.2 % for TChl *a* and from 33.6% to 27.4 % for PPig, while it is worsened for *H* and *N.*

The average precision (Table 48) remains comparable with results obtained in the previous exercise. The TChl *a* precision was 9.4 % (8.2 in HIP-2) and 12.2% for the PPig (same result obtained in HIP-2).

Table 47a. Absolute percent difference ($|\psi|_{Pi}^{L_j}$) for natural samples as a function of the method for PPig and MVChl *a*. In red, values higher than 25% (15% for the TChl *a*). *D* and *L* were assumed as reference quality subset.

$\|\psi\|$	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D-L subset	2.2	9.6	9.3	2.5	3.2	1.3	9.5	1.6	6.1	2.5	12.2	29.1	15.5	8.0
J	16.2	16.9	18.9	14.1	39.5	29.2	49.9	25.4	43.1	28.4	24.9	48.5	1.5	27.4
H	27.9	7.0	54.5	59.3	- -	54.7	25.7	51.3	44.9	57.6	- -	58.9	30.4	36.3
N	32.0	6.6	35.7	47.4	68.3	30.7	45.2	36.2	27.2	31.2	25.8	42.5	37.0	35.8

Table 48. Percent variation coefficient ($\xi_{Pi}^{L_j}$) for natural samples as a function of the method for PPig and MVChl *a*. The overall average for each pigment is given in the ***A*** entries.

ξ	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	13.2	0.0	0.0	8.7	5.9	6.4	12.0	21.4	13.5	7.0	7.9	22.3	6.1	9.9
L	4.2	5.1	3.6	4.0	2.9	2.5	18.4	3.4	6.8	4.4	4.5	4.1	4.6	5.3
J	6.8	15.2	10.1	10.5	20.5	7.5	29.9	19.0	16.6	7.1	9.2	18.1	6.9	14.2
H	5.0	8.6	11.3	17.8	- -	17.5	39.9	21.8	29.8	10.9	--	39.3	5.2	20.2
N	17.9	9.0	16.4	11.7	21.3	6.6	14.4	6.3	14.8	7.6	15.6	13.2	3.1	12.9
A	9.4	7.6	8.3	10.5	12.7	8.1	22.9	14.4	16.3	7.4	9.3	19.4	5.2	12.2

8.3.4 HIP-4 results of natural samples

Due the high and unexpected uncertainties found during HIP-3 on mixed standard comparison, the HIP-4 exercise was more focused on the preservation (i.e. storage phase) of the mixed standards and samples from the sampling to their distribution. The natural sample collection, preparation and distribution are those described in detail in Chapter 4.

A subset of 4 laboratories is established on the basis of the mixed standard results. Indeed, one of the laboratories in the subset, *N* only submitted 3 PPig for the natural samples: for this reason, *N* was not included in the subset for the PPig calculation. The average of the three laboratories *D*, *L* and *J* ($\overline{C}_{Pi}^{A^s}$ in Table 49) was considered as the true value for the computation of $\xi_{Pi}^{L_j}$ and $|\psi|_{Pi}^{L_j}$.

The average PPig uncertainties for the subset increase from 5.2% obtained for the mix-1 112 (Table 25), to 9.8% of natural samples (Table 50) while the TChl *a* uncertainties decrease from 9.7% obtained with the mix-1 112 standard, to the 6.2% for natural samples. In the previous HIP-1 and 2 exercises, the uncertainties were found to increase for TChl *a* in natural samples. However, this was not the case for HIP-4. It could be

attributed to a better homogeneity of the natural samples in comparison to the distributed mixed standards.

Table 49. HIP-4 PPig and MVChl *a* average values (in mg/m^3) for the replicates series. The overall average for each pigment for the reference subset is given in the $\overline{C}_{Pi}^{A^s}$ entries.

SAMPLE BATCH	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*
A	1.347	0.155	0.210	0.047	0.014	0.040	0.054	0.094	0.013	0.408	0.025	0.011	1.282
B	1.321	0.155	0.206	0.047	0.015	0.040	0.053	0.086	0.024	0.406	0.025	0.012	1.278
C	1.387	0.036	0.227	0.040	0.012	0.035	0.034	0.054	0.006	0.591	0.022	0.004	1.272
D	1.327	0.153	0.185	0.047	0.011	0.018	0.067	0.118	0.022	0.373	0.020	0.017	1.258
max	1.387	0.155	0.227	0.047	0.015	0.040	0.067	0.118	0.024	0.591	0.025	0.017	1.282
$\overline{C}_{Pi}^{A^s}$	1.346	0.125	0.207	0.045	0.013	0.033	0.052	0.088	0.016	0.445	0.023	0.011	1.273
min	1.321	0.036	0.185	0.040	0.011	0.018	0.034	0.054	0.006	0.373	0.020	0.004	1.258

The PPig subset precision ranged from 1.6% obtained for mix-1 112 (Table 26), to 6.7% in natural samples (Table 51). TChl *a* precision was 3.1% for natural samples, while it was 1.3% for mix-1 112.

Table 50. Absolute percent difference ($|\psi|_{Pi}^{L_j}$) for natural samples as a function of the method for PPig and MVChl *a*. *J, L* and *D* in the subset (*N* only submitted 3 PPig for the natural samples). In red, values higher than 25% (15% for the TChl *a*). The overall average for each pigment for the reference subset is given in the ***A*^{s}** entries.

$\lvert\psi\rvert$	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	8.9	22.5	12.3	1.7	5.7	8.8	2.6	5.7	16.5	3.3	21.4	4.8	2.6	9.5
L	7.3	8.4	3.3	4.6	7.1	1.8	6.1	4.9	14.8	3.6	28.3	6.6	2.6	8.1
J	2.3	14.0	18.3	4.8	12.4	10.2	6.5	8.8	26.9	7.9	24.7	6.0	8.8	11.9
N	0.0	0.0	82.0	0.0	0.0	0.0	0.0	0.0	0.0	2.2	0.0	0.0	13.8	7.0
E	44.9	29.1	9.6	100	14.6	32.9	22.9	24.0	23.1	19.1	33.2	25.6	- -	31.6
***A*^{s}**	6.2	15.0	11.3	3.7	8.4	6.9	5.1	6.5	19.4	4.9	24.8	5.8	4.7	9.8

Table 51. Percent variation coefficient ($\xi_{Pi}^{L_j}$) for natural samples as a function of the method for PPig and MVChl *a*. In red, values higher than 25% (15% for the TChl *a*). The overall average for each pigment for the reference subset is given in the ***A*^{s}** entries.

ξ	TChl *a*	TChl *b*	TChl *c*	Caro	ButFuco	HexFuco	Allox	Diad	Diat	Fuco	Per	Zeax	MVChl *a*	PPig
D	4.0	5.1	3.6	5.0	6.9	3.8	5.2	6.8	22.7	4.1	7.8	8.8	3.7	4.0
L	2.6	3.0	2.5	1.8	2.0	4.5	2.0	9.4	33.4	2.8	4.8	3.9	2.6	2.6
J	2.8	9.3	4.6	2.5	6.7	6.0	4.0	7.8	27.5	3.7	9.0	11.0	2.8	2.8
N	5.6	- -	82.0	- -	- -	- -	- -	- -	- -	- -	- -	- -	5.6	- -
E	34.8	16.8	5.3	- -	3.8	4.3	5.8	8.5	19.8	3.8	6.6	13.3	- -	34.8
***A*^{s}**	3.1	5.8	3.6	3.1	5.2	4.8	3.7	8.0	27.9	3.5	7.2	7.9	3.0	6.7

The secondary and tertiary pigments, reported by the three laboratories, *D*, *L* and *J,* are: Chlide *a*, MVChl *a* and Neo, Pras, Viol and Lut. DVChl *a* in natural samples was not

quantified by any of the laboratories. Although *N* only reported MVChl *a*, it was not considered in the quality subset. Results show that the uncertainties are higher for the secondary and tertiary pigments than for the PPig (Table 47 and 48) with the exception of MVChl *a*, that is comparable to results obtained for the PPig on natural samples. The Chlide *a* results were worse compared to those obtained for mix-1 112: the uncertainty rose from 53.4% for mix-1 112 (Table 27) to 79.1% for the natural samples (Table 52) and the precision (Table 53) decreased to 19.7% for the natural sample (previously 16.3% for mix-1 112, Table 28).

Table 52. Absolute percent difference ($|\psi|_{Pi}^{L_j}$) for natural samples as a function of the method for secondary and tertiary pigments. *D* did not report DVChl *a* for the natural samples (only in mix 107) and only reported the LOD for Pras. The overall average for each pigment for the reference subset is given in the ***A*^{s}** entries.

$\|\psi\|$	Chlide *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	39.5	2.6	16.8	12.9	21.4	12.4
L	100	2.6	12.6	14.8	16.0	16.2
J	79.2	8.8	28.8	3.2	5.5	9.7
N	- -	13.8	- -	- -	- -	- -
***A*^{s}**	79.1	3.0	19.4	10.3	14.3	12.8

Table 53. Percent variation coefficient ($\xi_{Pi}^{L_j}$) for natural samples as a function of the method for secondary and tertiary pigments. The overall average of each pigment for the reference subset is given in the ***A*^{s}** entries.

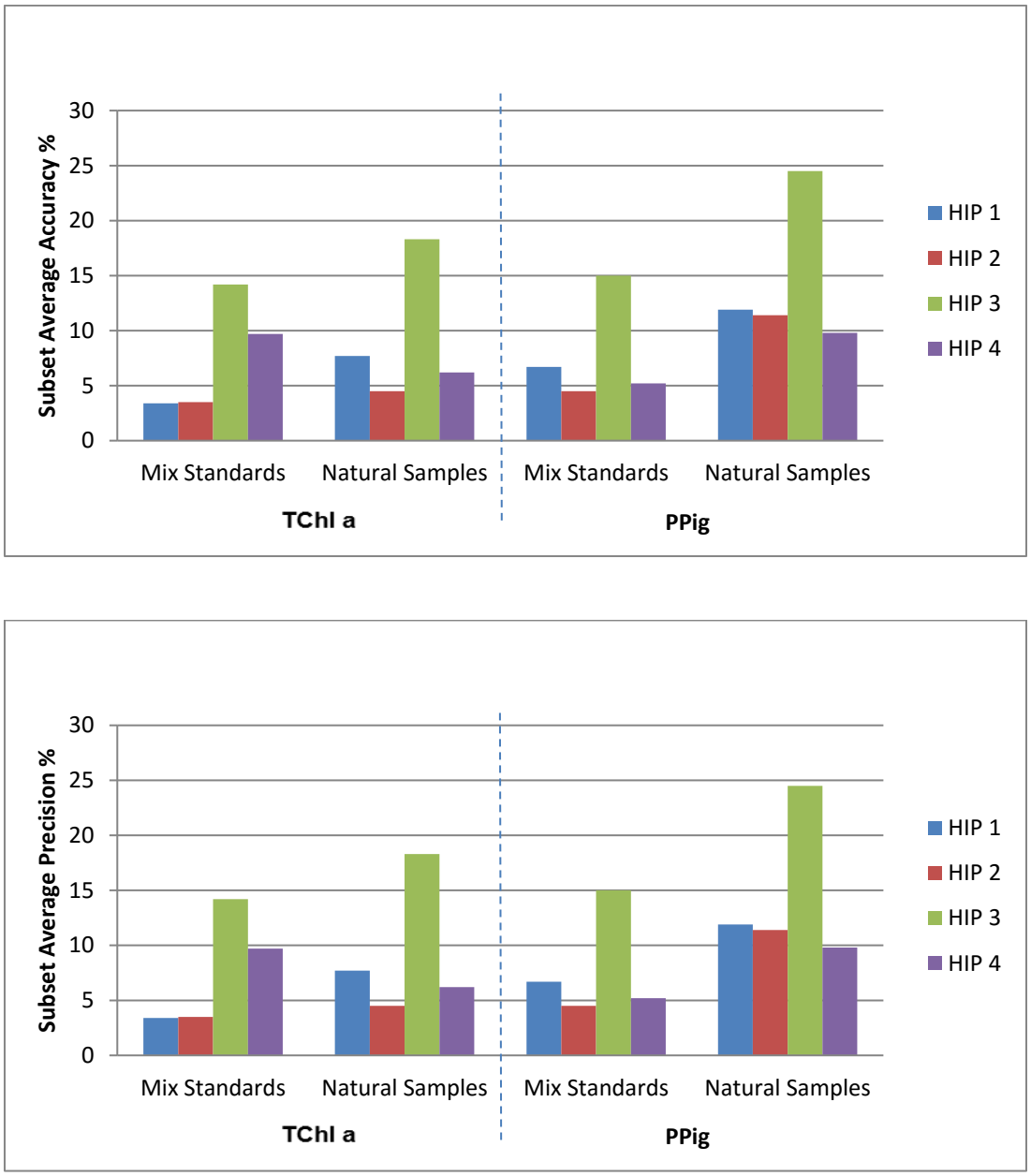
ξ	Chlide *a*	MVchl *a*	Neo	Prasi	Viola	Lut
D	9.9	3.7	4.6	0.0	12.9	13.7
L	10.7	2.6	4.8	0.0	10.4	12.7
J	38.5	2.8	5.5	0.0	9.6	10.9
N	- -	5.6	- -	- -	- -	- -
***A*^{s}**	19.7	3.0	5.0	0.0	11.0	12.4

9. Conclusion

The objectives of the HIP intercomparison exercises were *i.* to quantify single laboratory uncertainties for HPLC phytoplankton pigment measurements, *ii.* to quantify differences among European laboratories who apply published methods and finally *iii*. to create a reference community for HPLC pigment analysis in Europe.

The uncertainty calculation for phytoplankton pigment measurements was carried out over a 3-step evaluation that allows the laboratory issues to be identified. The source of poor performance could be laboratory practice, it could be related to the analytical instrumentation in use or to the sample extraction procedure. By going through these different steps and creating each time a reference subset that provided the true value for the pigment concentrations, it was possible to identify weaknesses in methods and procedures, to improve or maintain the quality of results for a single laboratory, and to trace and document with time the condition of a single laboratory with respect to the others. This work forms a picture of the State of Art of European Accredited or Reference Laboratories for HPLC pigment analysis during the past 7 years.

Fig. 4. Uncertainty and precision evolution through the HIP exercises (data refer to the reference subset)



The decision to create a subset based on the exclusion criteria of 25% for PPig and 15% TChl *a*, does not affect one methodology with respect to another. It confirms that good laboratory performance is independent from the implemented method, when the method is an assessed and published method and a good laboratory practice has been applied. In the case of HIP-3, when it was not possible to establish a consistent subset based on the mixed standard analysis, the definition of a true value for the pigment concentrations was difficult and the final uncertainties were very high for all laboratories (Table 55). During the other HIP exercises, when the three-step evaluation could be performed, laboratories that qualified poorly in the performance metrics, did not obtain good results even in the following steps and often were excluded from the laboratory subset. This was the case for *E* laboratory that qualified 'semi-quantitative' in its analytical performance. This was reflected in an overall mediocre performance (associated to high uncertainties). The problems reported by laboratory *E* confirm the validity of this approach.

If all the HIP exercises are considered as a whole, a coherent picture of the uncertainties related to phytoplankton pigments measurements over the past 7 years can be delineated.

Values reported in Fig. 4 refer to the TChl *a* and PPig for the established reference subsets for both mixed standards and natural samples. The uncertainty of TChl *a* for natural samples is less than 8% for all the exercises, with the exception of HIP-3 (18.3 %), when no reference subset could be established. The threshold required for data satellite validation and algorithm refinement (15% of uncertainty) is well achieved (Fig. 4) within all the HIPs.

It is noteworthy that, the results obtained with the mixed standards were not always better than the results on natural samples (see the HIP-4 results).

During each HIP exercise, it was possible to focus on problems that had emerged during the previous HIPs. This helped the laboratories involved to become more confident with their methods. *D* and *L* could focus on the extraction problems originating from the different filter sizes. *N* could familiarize with the Performance Metrics evaluation and revise its data calculation, resubmitting results in HIP-2 and test the newly implemented Van Heukelem method in HIP-4. *C* and *E* also had the opportunity to validate newly implemented methods.

The main achievement was definitively the creation of a reference community at a European level for finding solutions to new emerging problems on HPLC phytoplankton pigment analysis. This also forms a comprehensive picture, in time, of the state of art of the reference and accredited European laboratories performing phytoplankton pigment analysis.

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List of abbreviations and definitions

Methods

D DHI, Danish Institute for Water and Environment, Denmark

L LOV, CNRF *Laboratoire d'Oceanographie de Villefranche*, France

J JRC, Marine Laboratories, Joint Research Center, European Commission

C CIMA, Centre for Marine and Environmental Research - University of Algarve

N NIVA*,* Norwegian Institute for Water Research, Norway

H HZG, *Helmholtz-Zentrum Geesthacht*, Germany

E ENEA, Italian National Agency for New Technologies, Energy and Sustainable Economic Development, Italy

Symbols

$\hat{A}_{Pi}$ The peak area of pigment P_i

$\hat{A}_c$ The peak area of the internal standard in the extraction solvent

$\hat{A}_s$ The peak area of the internal standard in the sample

A The average of all the laboratories

A^s The average of the laboratories reference subset

$\overline{C}_{Pi}^{A}$ The average concentration of each pigment of each batch across the laboratories

$\overline{C}_{Pi}^{A^s}$ The average concentration of each pigment of each batch across the laboratories reference subset

$\tilde{C}_{Pi}^{L_j}$ The single pigment concentration as function of laboratory

$\overline{C}_{Pi}^{L_j}$ The average concentration over the batch triplicates analysis performed by each laboratory

L_j The laboratory or method code

LOD_{eff} The limit of detection

N_L The number of the laboratories quantifying the pigment

N_R The number of replicates

R_{Pi} The response factor for pigment P_i,

$\widehat{R}_S$ The minimum resolution determined from a critical pair for which one of the pigment is a primary pigment

S_k The sampling station or sapling batch

t_R The retention time

V_c The volume of sample extract injected onto the column

V_f The volume of water filtered for each sample

V_x The extraction volume;

$\overline{\xi}$ The average percent variation

$\overline{\xi}_{inj}$ The injection precision

$\xi_{Pi}^{L_j}$ The percent variation coefficient of pigment as function of the laboratory

$\overline{\xi}_{tR}$ The average variation coefficient of the retention time

$\overline{\xi}_{cal}$ The average variation coefficient for calibration of dilution devices

$\sigma_{Pi}^{L_j}$ The standard deviation of each pigment for each laboratory

$\Psi_{Pi}^{L_j}$ The average of the relative percent differences

$\left|\overline{\psi}\right|$ The average of the absolute percent differences

$\left|\psi\right|_{Pi}^{L_j}$ The absolute unbiased percent difference for each single pigment as a function of the method

$\left|\overline{\psi}\right|_{res}$ The average of the absolute residual

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Annexes

Annex I. LOV participant comments to HIP-1 exercise

IMPORTANT NOTE: Problems encountered during this exercise:

1 The results from the sequence of the 30/9/09 had to be discarded because of sudden degradation of the chromatographic column.

2 The extracts from the 30/10/09 were maintained at -80°C until the 20/10/09 when reanalysis took place after a change of column.

3 The reason for the degradation seems to originate from the TBAA mixture used as buffer which could not be maintained at 4°C due to a faulty refrigeration system.

4 The results from the 29/9/09 were acceptable but degradation of the chromatography did become slightly visible towards the end of the sequence.

5 The extracts from the 29/9/09, also maintained at -80°C were reanalysed on the 26/10/09 after the change of column.

6 The new column installed has not been calibrated, but shows a clear improvement in performance compared to the previous one and in general final concentrations are comparable.

7 C samples were also reanalysed on the 16/11/09 along with the samples which were sent to Villefranche at a later stage

8 The DHImix 106 injections were done after the column change

9 DHImix 106: injections 07, 08 and 09 were done with a volume of 125 µL. The rest was done with 60µL because of peak deformation of the early eluting peaks due to solvent effect

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